Endolithic Microbial Communities in Dolomite

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2010

Acknowledgments

I am very grateful to Reinhard Bachofen for his enormous patience with me and allowing me to investigate the hidden microbial life in dolomite stone from the Piora Valley. His supervision and advice was crucial for this PhD work. I thank him very much for putting complete confidence in me and for his strong support not only during difficult times.
I am very grateful to Jakob Pernthaler to "adopt" me and to represent my thesis in front of the faculty committee. I also thank him very much for reviewing my thesis.
I am very thankful to Leo Eberl for being in my graduation committee and reviewing my thesis.
Many thanks go to Thomas R. Neu for his help and supervision to analyze our "living stone" with laser confocal microscopy. Very thankful I am to Judith Blom for her supervision and help with the HPLC analysis of the endolithic band of the Cadagno dolomite. I thank Ferdinand Schanz and Markus Wiggli for fruitful discussions and the help in processing the data of the in vivo spectra. The excellent technical assistance of Ute Kuhlicke (LSM) and Urs Jauch (SEM) is deeply appreciated.
I thank very much Rudolf Amann and his crew (especially Jörg Wulf) for introducing me into the technique of Fluorescence *In Situ* Hybridization (FISH) and Catalyzed Reporter Deposition Fluorescence *In Situ* Hybridization (CARD-FISH), and I would like to thank Wolfgang Ludwig and Frank Oliver Glöckner for broadening my skills with the program "ARB".
Further I am indebted to Leo Eberl, Beat Keller, Ueli Grossniklaus, Friedrich Jüttner, Kurt Hanselmann, Robert Dudler, Elena Conti, and Jakob Schneller, for being allowed to use their facilities and instruments for my work.
I am also indebted to several students who worked during courses on endolithic material.
Beautiful memories I share with many former and current lab members of the two institutes at the Botanical Garden in Zürich but especially with those who were temporarily always around me like Rolf Stettler, Philipp Bosshard, Yves Santini, Marcello Marchiani, Maja Lazzaretti, Anne Postec, Christine Lehmann, Janine Kessi, Christof Eichenberger, Sandra Scherrer, Amal Joseph Johnston, Olga Kirioukhova, and of course Munti Yuhana.

Finally, I am grateful to my parents Erwin and Helga, for bringing me up, educating me, and supporting me whenever there is need for it.

Financially I was supported by the Canton of Zürich through the University of Zürich, the Swiss National Science Foundation and by the Zürich Limnology-Hydrobiology Foundation for Aquatic Research.

Summary

Subsurface microbiology has been a rapidly growing field within microbial ecology in the past decades, covering interest in places such as nuclear waste disposal sites and the surface of Mars. This study focuses on a community of endolithic microorganisms predominated by phototrophs forming a distinct grayish-green band a few millimeters below the surface of bare dolomitic rocks in the Piora Valley in the Swiss Alps.

Using *in situ* reflectance spectroscopy, chlorophyll a (Chl *a*), phycobilins, carotenoids, and an unknown type of bacteriochlorophyll-like pigment absorbing *in vivo* between about 712 and 720 nm were detected. High-pressure liquid chromatography analyses of pigments extracted with organic solvents confirmed the presence of two types of bacteriochlorophylls besides chlorophylls and various carotenoids. Spherical organisms of varying sizes and small filaments were observed *in situ* with scanning electron microscopy and confocal laser scanning microscopy (one- and two-photon technique). The latter allowed the visualization of the distribution of phototrophic microorganisms by the autofluorescence of their pigments within the rock. Application of fluorescence-labeled lectins demonstrated that most cyanobacteria were embedded in an exopolymeric matrix. Based on small subunit ribosomal RNA gene sequences, a diverse community driven by photosynthesis has been found. *Cyanobacteria* (57 clones), especially the genus *Leptolyngbya*, build the functional basis for this endolithic community which contains a wide spectrum of so far not characterized species of chemotrophic *Bacteria* (64 clones) with mainly *Actinobacteria*, *Alpha-Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*, as well as a cluster within the *Chloroflexaceae*. Furthermore, a cluster within the *Crenarchaeotes* (40 clones) has been detected. Although the eukaryotic diversity was outside the scope of the study, an amoeba (39 clones) and several green algae (51 clones) have been observed. We conclude that the bacterial diversity in the investigated dolomite in the Piora Valley is considerable and that *Archaea* are present in this endolithic habitat as well.

Zusammenfassung

Die "Mikrobiologie unter der Oberfläche" hat innerhalb der mikrobiellen Ökologie in den letzten Jahrzehnten immer mehr an Bedeutung gewonnen. Sie befasst sich mit Bereichen von der unterirdischen Abfallentsorgung bis hin zur Suche nach Leben auf anderen Planeten. Die vorliegende Arbeit konzentriert sich auf die Untersuchung einer endolithischen Mikroorganismengemeinschaft, die überwiegend aus phototrophen Organismen besteht, ein ausgeprägtes grau-grünliches Band bildet und sich wenige Millimeter unter der Oberfläche von nackten, exponierten Dolomitfelsen im Piora-Tal in den Schweizer Alpen befindet. Mit Hilfe von *in situ* Reflexionsspektroskopie wurden Chlorophyll *a* (Chl *a*), Phykobiline, Karotinoide und eine unbekannte Art eines bakteriochlorophyll-ähnlichen Pigments, das *in vivo* bei etwa 712 bis 720 nm absorbiert, detektiert. Hochdruck-Flüssigkeitschromatographie (HPLC) von mit organischen Lösungsmitteln extrahierten Pigmenten bestätigte das Vorhandensein von zwei Typen von Bakteriochlorophyllen neben Chlorophyllen und verschiedenen Karotinoiden. Kugelartige Organismen von verschiedener Grösse und kleine Filamente wurden *in situ* unter Anwendung von Rasterelektronenmikroskopie (SEM) und konfokaler Laser-Rastermikroskopie (Ein- und Zwei-Photonen-Technik) (CLSM) beobachtet. Aufgrund der Autofluoreszenz ihrer Pigmente ermöglichte die konfokale Laser-Rastermikroskopie die Sichtbarmachung der Verteilung von phototrophen Mikroorganismen innerhalb des Gesteins. Die Anwendung von fluoreszenz-markierten Lektinen zeigte, dass die meisten Cyanobakterien in einer Matrix von exopolymeren Substanzen eingebettet sind. Basierend auf Sequenzen des Gens der ribosomalen Ribonukleinsäure der kleinen Ribosom-Untereinheit wurde eine vielfältige Gemeinschaft gefunden, die ihre primäre Energie zum Leben aus der Photosynthese gewinnt. Cyanobakterien (57 Klone), speziell die Gattung *Leptolyngbya*, bilden die funktionelle Basis für diese endolithische Gemeinschaft, die ein breites Spektrum an bisher nicht charakterisierten Arten von chemotrophen Bakterien (64 Klone) mit hauptsächlich *Aktinobakterien*, *Alphaproteobakterien*, *Bakteroideten* und *Azidobakterien*, sowie auch eine Gruppe innerhalb der *Chloroflexazeen* umfasst. Zudem wurde auch eine Gruppe innerhalb der *Crenarchäen* (40 Klone) nachgewiesen. Obwohl die eukaryotische Diversität nicht zum Untersuchungsgegenstand gehörte, wurden auch eine Amöbe (39 Klone) und mehrere Grünalgen (51 Klone) erfasst. Zusammenfassend lässt sich schlussfolgern, dass die bakterielle Diversität im untersuchten Dolomitfelsen im Piora-Tal beträchtlich ist und in diesem endolithischen Habitat auch Archäen (Archebakterien) vorkommen.

Curriculum Vitae

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Preface

“Understanding the amazing diversity of life forms on Earth” is one of the guidelines for the present thesis [from “Some Things Are Better Left on Mars” by Olivia Judson in The New York Times. April 19th, 2004]

1. Introduction

1.1. Definition and history

Endolithic microorganisms – or shorter "endoliths" – are organisms that live inside of rocks. They are found in a wide range of rock environments: from their surface to kilometers beneath the subsurface and also in subaqueous rock systems. Many of these organisms are extremophiles, living in places previously thought to be inhospitable to life. They are of particular interest to astrobiologists, who theorize that endolithic environments on Mars and other planets constitute potential refugia for extraterrestrial microbial communities. (Adapted from Wikipedia).

Our knowledge that microorganisms inhabit not only the surface but also deep zones of the crust of the earth and deep sea sediments, arose only in the mid of the last century. Lipman (1928) was one of the first to come up with investigations about the presence of microbes in subsurface rocks: "I have also discovered other types of micro-organisms in a Pliocene rock which derives from a depth of several hundred feet from which it has recently been uncovered." Zobell (1946, 1964) studied the microbiology of deep sea sediments and of boreholes for water and oil prospection. Russian scientists pioneered the microbiology of oil fields and permafrost (Ginsburg-Karagitscheva, 1933; Issatchenko, 1940; Elazari-Volcani, 1943; Kuznetsov, 1962). Subsurface microbiology has its roots in different disciplines, combining microbiology, geology and hydrology. Recently deep subsurface microbiology was stimulated by the search for a safe deposition of radioactive waste (Bachofen, 1998). The present research in subsurface microbiology focuses more on general microbial ecology and biogeochemistry of continental and deep sea sediments, arctic permafrost and groundwater aquifers. Only recently were methods developed to

uncover the richness in number and biodiversity of the subsurface. Following the calculations of Whitman et al. (1998) 94% of the microbial biomass is in the subsurface.

Table 1: Number and weight of bacteria on earth (Whitmann et al., 1998, modified)

habitat	Number of cells, x 10^{28}	Toal carbon, kg x 10^{12}
Aquatic systems, rivers, lakes, sea	12	2.2
Subsurface of oceans	355	303
Terrestrial systems, soil	26	24
Terrestrial subsurface	25 - 250	22 - 215
total	415 - 640	353 - 546

As comparison: total carbon of the whole terrestrial vegetation worldwide amounts for 560 kg x 10^{12}

The research in subsurface microbiology was intensified a few decades ago due to the urgent need to understand the microbial processes in polluted soils and aquifers (Ghiorse, 1997). Energy and defense related activities have led to a sum of contaminations in the subsurface. If microbes were present and metabolically active in the whole subsurface, they could be harmful by dispersing the contaminants and accelerating the release of toxic material into the biosphere (Fredrickson and Onstott 1996; Fredrickson and Balkwill 2006). With classical methods used at the beginning of this research, the presence, abundance, diversity and the spatial distribution of these microorganisms in the subsurface was demonstrated. Up to about 1980 it was not possible to collect subsurface samples aseptically, thus contamination could not be excluded. Nevertheless it became clear that sterility did not start shortly beneath the soil surface. The presence of bacteria has been shown in subsurface layers located several kilometers deep, their number, however, decreased with gaining depth by several orders of magnitude (Colwell et al. 1997; Onstott et al. 1998; Orphan et al. 2000; Moser et al. 2005; Sahl et al. 2008).

The number of cultivable or viable cells depends on the type of rock material; it drops with depth even more than the total number of cells. Biological processes at these great depths are rather slow, as they are often limited by the scarcity of the nutrients and the energy source available. Additionally, at a certain depth, the increasing temperature also becomes a life limiting factor (Kerr, 1997). The actual record of heat tolerance stays at about 121℃ (Kashefi and Lovley, 2003) or 122℃ under a pressure of 200 to 400 bar (Takai et al 2008) which would be equal to the conditions at about 5 km beneath the surface (Kerr, 1997).

The habitat of endoliths in the Deep Subsurface can be divided roughly into two types: sedimentary deposits which may contain organic carbon and igneous rocks which

are typically devoid of organic matter and have been subjected to high temperature and/or pressure at some point in their history. In igneous rocks water typically flows via fractures since most crystalline rocks lack pores of sufficient size and interconnectivity for water flow or to provide space for microorganisms, as it is the case in sediments (Fredrickson and Balkwill 2006). Chandler and coworkers described the prokaryotic diversity of a deep subsurface paleosol with a low biomass content at the US Department of Energy's "Hanford Site" in south-central Washington State. Besides organisms like *Pseudomonas, Bacillus, Micrococcus, Clavibacter, Nocardioides, Burkholderia, Comamonas*, and *Erythromicrobium* they also found organisms which cluster with the *Chloroflexaceae* and with the *Crenarchaeota* (Chandler et al. 1998)

The development of modern molecular methods during the past 20 years allows now to obtain more detailed information on microbial diversity and heterogeneity of subsurface habitats, on the structure of these microbial communities and on the phylogeny of the organisms.

The definition of endolithic microorganisms in Wikipedia given at the beginning is rather broad and covers the whole subsurface. Our focus will concentrate more on a subgroup of the endolithic habitat, namely the porous space a few millimeters below the surface of rocks.

1.2 Early descriptions of epi- and endolithic microorganisms

Microorganisms living in pores and cracks of rocks were first described more than a century ago. One of the pioneers, Schroeter (1908), reported about observations in the Alps, the Churfirsten, Säntis, and Scheienfluh mountains (2'625 m a. s. l. 47°0′36″ N, 9°52′15″ E, "Rätikon") and mentioned rock destroying, chemolithotrophic bacteria such as "Salpeterbakterien" or "Nitromonades" and cyanobacteria – then called "Spaltalgen" – with a gel-like envelope such as found around *Gloeocapsa* and *Stigonema*. Later, Diels (1914) reported on the alpine vegetation of the Dolomite Mountains in the South Tyrol in the region of the Tschamin Valley at altitudes between 1000 and 2500 meters above sea level, focused on south exposed naked rock walls. A chemical analysis resulted in a rock composition of around 30% Ca^{2+}, 21% Mg^{2+}, 47% CO_3^{2-}, with traces of iron, manganese, water and "Gangart" (Quartz, Calcite, Dolomite, Barite, and Fluorite). This is close to the "Standard Dolomite" containing 54.2% $CaCO_3$ and 45.8% $MgCO_3$ (Zirkel, 1893). The

temperature of the rock inside and the zone closely above it varied significantly over the day. The stone and especially the air near its surface were – depending on the time of the day and the direct sun radiation onto the rock wall – about 3° to 7° C warmer than the air half a meter apart. Examining the rock flora, he used the term "real lithophyts" for plants that are able to colonize naked rock. According to Diels (1914), in the European temperate zone, only the cryptogams, lower plants such as ferns, mosses, algae, fungi, and lichens, can fulfill this task. He divided them in the two classes, **epiliths** and **endoliths**. Epiliths are living at or on the surface of the stone and are fully exposed to air and light, while endoliths grow below the rock surface and receive air and light only in much reduced quantities.

Illustrations showing the different types of lithic habitats have been given by Golubic et al., (1981) and Cockell et al., (2005) (Fig. 1).

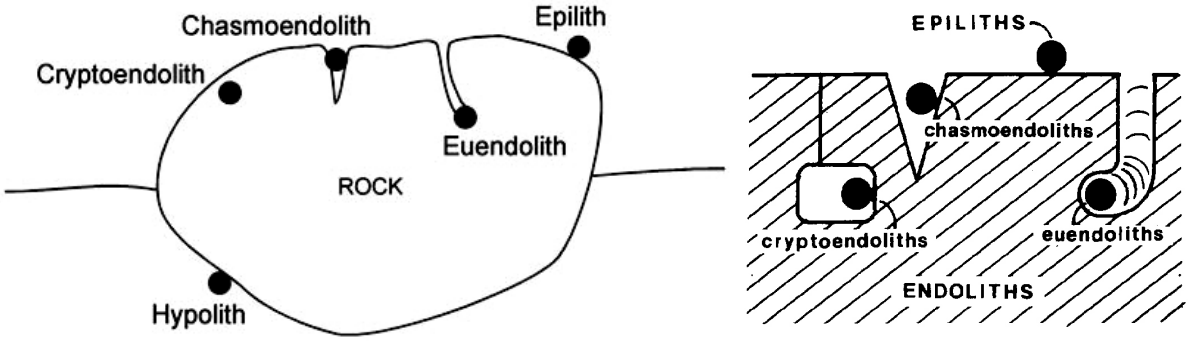

Fig. 1. Lithic habitats, after Cockell et al. (2005) (left) and Golubic et al. (1981) (right).

Golubic, Friedmann, and Schneider (1981) subdivided the endolithic organisms further into:

cryptoendolithic:	Habitat in structural cavities within porous rock (microbes are fullyenclosed within porous rock) (crypto- = hidden)
chasmoendolithic:	Habitat in fissures and cracks within the rock (microbes adhere tosurfaces along fissures) (chasm = cleft)
euendolithic:	Habitat is formed with active boring / penetration by themicroorganisms leaving tunnels that conform with the shapes of their bodies (eu = good, true)

foot-note

The Greek word ενδον (endon) means "inside", "indoors", "at home". Λιθοζ (lithos) stands for "stone", "boulder", or "rock". "Endolithic" therefore means "inside the rock". The preposition Επι (epi) means "on", "upon", "above", "close to", or "at". An epilithic organism is therefore living on the surface of a stone, like lichens. Υπο (hypo) stands for "below", "sub-", "under", "beneath".

Most of the time it is difficult to distinguish between chasmoendolithic and cryptoendolithic organisms, because it is hardly possible to determine whether the microorganisms are located in fully closed cavities or just in small pores and gaps of the rock. For euendolithic ones, it would be technically even more difficult to find whether active boring took place.

Most of the rock surfaces lack higher vegetation and have a bright reddish to yellowish color (Diels, 1914). This is interpreted as “Rinden” formation” (bark or rind formation) by the deposition of lime stone, a process stimulated by phototrophic organisms. The reddish color is attributed to traces of ferric iron hydroxide [$Fe^{3+}(OH)^{-}_{3}$]. Rind material effervesces upon contact with hydrochloric acid whereas the inner dolomite rock material hardly does at ambient temperature.

In the Schlern Dolomite, the first colonizers are *Schizophyceae* or Spaltalgen, now named *Cyanobacteria*. They often form the so called "Tintenstriche" ("Ink Stripes") at sites where water periodically trickles over the rock. Diels (1914) listed as **epilithic** organisms members of the *Gloeocapsaceae* such as *Cyanocapsa*, *Chrysocapsa*, and *Xanthocapsa*, and members of the *Eugloeocapsaceae* with a colorless sheath. Occasionally, he detected dense, coriaceous, stringy, lichen like nodes of brown black color: "The content of the cells during August was mostly glycogen and had a bright brownish color." Depending on whether these polysaccharides were found also outside of the cells, Diels may already found a kind of extracellular polymeric substances (EPS).

Larger associations of *Cyanocapsae* or *Xanthocapsae* had different types of gelatinous mantles as earlier found by Nägeli (1849) and Brand (1900) and confirmed by Diels. When the cells were sun-exposed, the gelatinous layer is stained, whereas in the shadow it is transparent. According to Diels, this holds true also for the single cells: The sun-exposed cell wall is dark; the cell wall on the other side is transparent. Diels confirmed the existence of three different states of the frequently found cyanobacterium *Gloeocapsa alpine* (Brand, 1900). The stained state (status coloratus), the dry state (status siccus) and occasionally the hibernating state (status perdurans) are found next to each other. Normally, the air exposed layers of a thick colony are in the dry state while the inner ones are in a more hydrated state. When films of *Cyanocapsae* and *Xanthocapsae* get denser, the typical vertically oriented "Tintenstriche" (Ink Stripes) become visible on the rock surface.

Foot-note

chasmoendolithic (Greek χασμα, χασματο = gaping aperture, chasm, earth's maw),

cryptoendolithic (κρυπτοσ = hidden, buried, covered, clandestine, secret)

With denser cyanobacterial vegetation, besides *Gloeocapsae,* various filamentous microorganisms like species from the genus *Scytonema* are found, mainly *Scytonema crassum* or *Scytonema densum* (Diels, 1914).

In contrast to these epilithophyts, **endolithophyts** live in pores, cracks and fissures of various sizes, the smallest ones are not seen with a magnifying glass. Such fine structures had been described by Doelter and Hoernes (1875) as "The rock is formed of an aggregate of unnumbered small rhombohedra, which touch each other only at single points."

Many of these fine fissures and cracks close to the surface of the rock are inhabited by phototrophic organisms, forming a colored band of endolithic organisms. "Sehr viele dieser feinsten Spalten sind in ihren unweit der Gesteinsaussenfläche gelegenen Partien bewohnt von Algen, welche eine als endolithisch zu bezeichnende Formation bilden." (Diels, 1914, p.514). Oettli (1905), studying lime stone in the Churfirsten mountains in North-East of Switzerland,found a "lively chlorophyll green spot" below almost every lime stone surface when hit with a hammer.

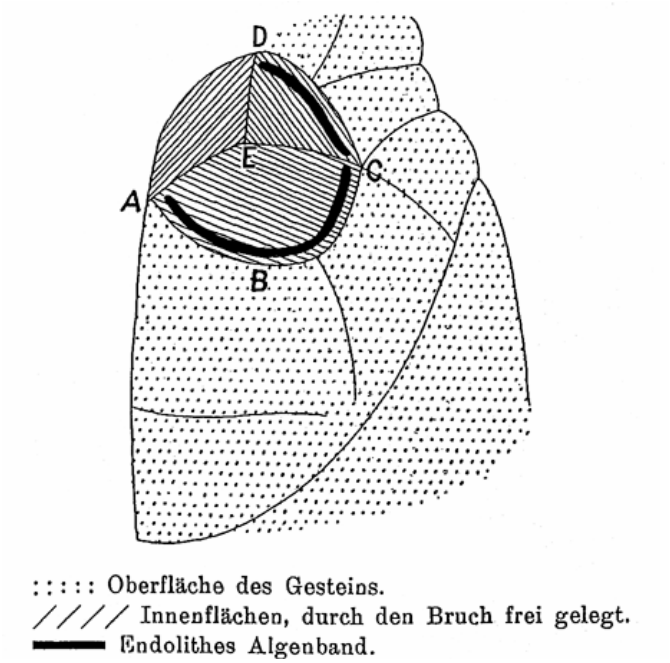

Fig. 2.: Scheme of a piece of rock surface, where the part A to E has been removed by a stroke with a hammer in order to show the endolithic bands (Diels, 1914).

Diels enlarges these observations describing the endolithic band formed by the green microorganisms (Fig. 2) and stating that these endoliths behave differently from endolithic lichens or drilling algae as they do not start from the rock surface nor do they remain in contact with it like lichens when they form the apothecia. Thus, below the surface of the

rock, the smallest cracks are colonized by a typical microbial vegetation not visible from the outside. Their dwelling region starts at a depth of about 2 to 4 mm and reaches down to about 8 mm in case the rock surface is bare outside. In rocks covered with epilithic vegetation, the endolithic zone approaches the surface up to 2 or 1 mm (Diels, 1914).

The most numerous and widest dispersed species among endolithic microorganisms is a small cyanobacterium, *Gloeocapsa* (from Greek *gloia*, "glue" and Latin *capsa*, "box"), with a thick, hardly layered gel-like envelope, and a distinct boundary of a differentiated membrane, the cuticula (Brand, 1900). The bluish-green cell has a diameter of 0.8 to 1.6 µm, with the gelatinous envelope up to 4 to 6 µm in thickness. The color of the cell is of variable intensity and quality. Jaag (1945) found that it even works as an indicator of the environmental pH. Both, cell and envelope get easily dyed by gentian violet and the outer membrane of the gelatinous envelop resists to 50% chromic acid for a “very long time” (Diels, 1914).

During the 1st half of the 20th century the study of endolithic organisms was fully restricted to light-microscopy, thus mostly colored organisms, that is phototrophic ones, were described. Diels admits that it is difficult to distinguish and identify correctly the various forms of *Chroococcaceae in situ*, even with long experience or after preparation of pure cultures on artificial media. Clumps of cells are often nested twice or more times but contain only two or four cells per pack. Nevertheless the number of cells can increase rapidly during growth, leading to clumpy aggregates of undefined shape. Jaag (1945), pioneering the description of cryptogam flora on and in rocks, presents color drawings of such *Gloeocapsa sanguinea* aggregates (Fig. 3), a morphology already described earlier by Nägeli of various *Chroococcus* species (1849) (Fig. 4).

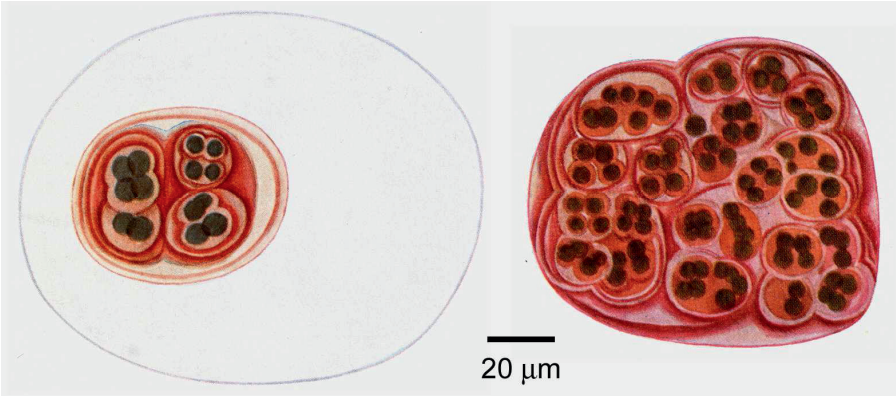

Fig.3. *Gloeocapsa sanguinea* (Ag.) Kütz. sensu. nostro. In the "status familiaris lamellosus, coloratus, Ralfsianus" (left) and a "status familiaris lamellosus, coloratus, typicus (right). The transparent circle around the cells is the gelatinous envelope, here reaching 140 µm in diameter. Pigmented dark single cells have a diameter of 4 to 8 µm. The substrate is acidic (below a pH of 6.5) (Jaag, 1945).

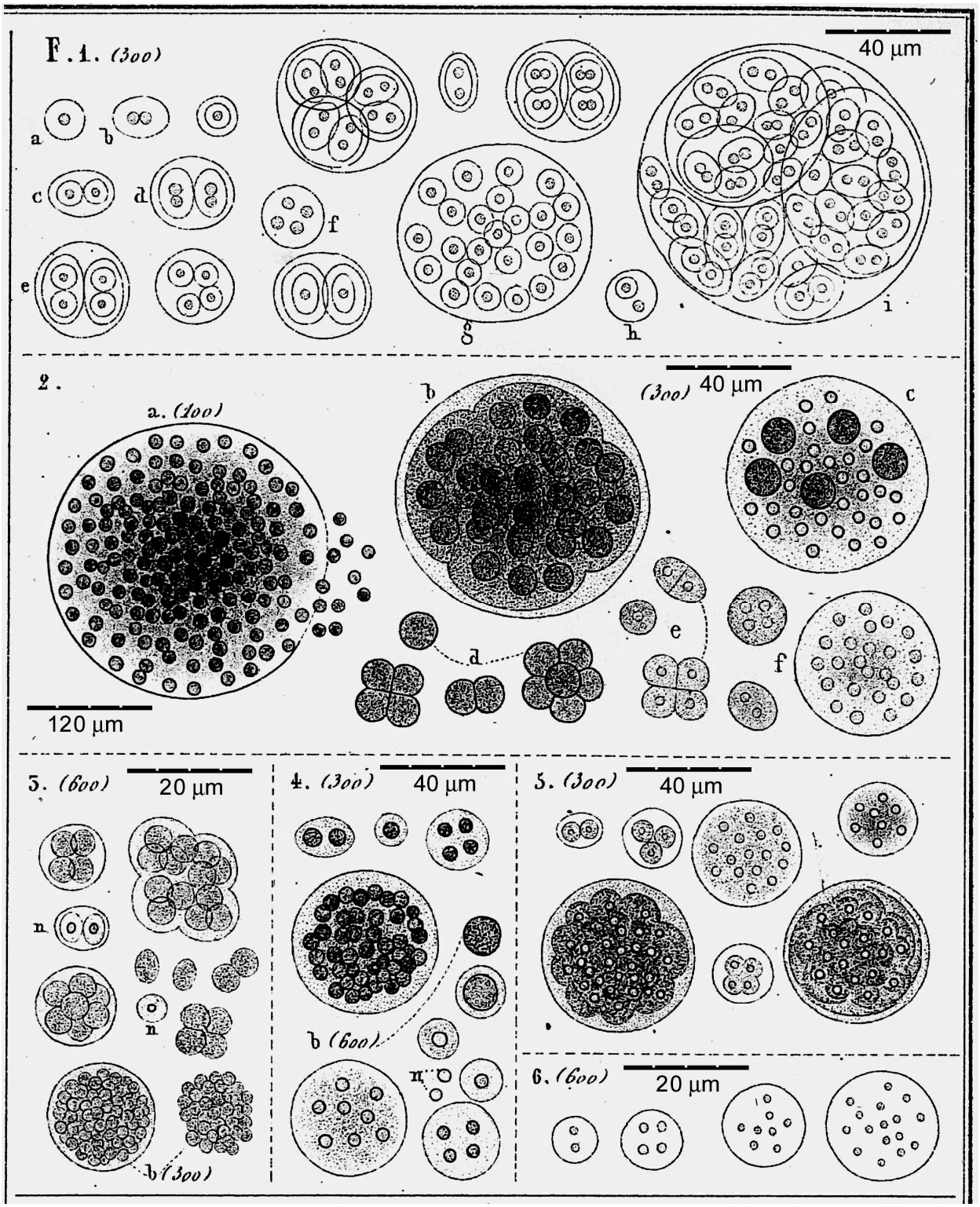

Fig. 4. Several species of *Gloeocapsa*: *G. atrata* (F.1.), *G. opaca* (F.2.), *G. ambigua a. fuscolutea* (F.3.), *G. ambigua b. violacea* (F.4.), *G. ianthina* Kg (F.5), and *G.punctata* (F.6.). In brackets the magnification adjacent scale bars are introduced (Nägeli, 1849)

The most numerous *Gloeocapsa* species in the Dolomites of the South Tyrol resembles best *Gloeocapsa punctata* Näg. Often it is possible to find pure colonies of one species. There is a range of other forms of *Gloeocapsae* with verdigris green cells and a thick, often colorless, mostly not layered, sometimes very clearly layered gelatinous envelope.

Strains of *Gloeocapsae* differ in color and in size, but also by the proportions of the cells to their envelopes. The cells measured between 2 and 5 µm, clumps can reach 25 µm or more.

One of the oldest studies on bacteria and algae goes back to Agardh (1824) who wrote a book on the systematics of algae, “Systema Algarum”. *Trentepohlia, Scytonema, Stigonema, Oscillatoria, Calothrix, Lyngbya* and other genera that are common in and on rocks are described, e.g. ”*Protococcus viridis* - globulis, viridibus. Ad muros vulgaris” – "spherical, green, on walls common”, possibly the first description of epilithic *Gloeocapsa*. Up to the middle of last century, several specific studies about microbial life in and on rock were written by Bachmann (1890), Brand (1900), Oettli (1905), Fritsch (1907), Schroeter (1908), Diels (1914), Bristol (1919), Blöchliger (1931), and Jaag (1945).

1.3. The rock environment, endolithic habitats, and EPS

There are different types of matrices where endoliths are found. The material ranges from quite soft dolomite over limestone (calcite: Mohs hardness 3) to sandstone and granite (quartz: Mohs hardness 7). In granite, fissures allow chasmoendolithic distribution, in softer porous stones, we find cryptoendolithic growth. Fissures and pores offer large surfaces where the organisms may form a biofilm, which consists of layers and aggregates of cells, embedded in exopolymeric substances, also called extracellular polymeric substances (EPS) or glycocalyx (Costerton et al. 1981, Donlan and Costerton, 2002; Staudt et al., 2004). These are high molecular-weight, carbon-rich molecules released mainly by bacteria and microalgae (Kumar et al., 2007, Murray et al. 2002, Underwood and Paterson, 2003). For endolithic organisms extracellular polymeric substances are extremely important for survival; they represent a substantial fraction of the total production of organic compounds. In the phytoplankton on average 10–20% of net primary production goes into the EPS (Baines and Pace, 1991), more than 40% in benthic algal communities (Underwood and Paterson, 2003) and possibly up to 65% in riverine sediments (Gerbersdorf et al., 2009, assuming that 1 bacterium has a weight of 5 pg in a sample from 1cm depth at Fahlberg, Elbe river). The chemical composition of the EPS matrix varies depending on its organismic origin and the local environment, as EPS-embedded microcolonies show widely differing lectin binding properties (Horath et al. 2006).

EPS contain polysaccharides (40–95%), proteins (up to 60%), and minor traces of nucleic acids, lipids, and other biopolymers (Flemming and Wingender, 2001a; Branda et al., 2005; Flemming et al., 2007; Ma et al., 2009). Polymeric substances fulfill many functions for the embedded microorganisms including attachment of the cells or locomotion, protection from desiccation, resistance to toxins, stabilization of the structure, ion sorption and exchange, and enhancement of their ability to store nutrients (Decho, 1990; Flemming et al., 2007). In marine coastal ecosystems EPS support aggregation, increase sediment stability, adsorb and modify contaminants, and provide also a food source for invertebrates (Bhaskar and Bhosle, 2006; Decho, 1990; Passow, 2002; Paterson et al., 2008). In phytoplankton, growth of benthic diatoms and their production of transparent exopolymer particles (TEP) correlate (Passow 2002, Perkins et al., 2001; de Brouwer et al., 2005). However, Gebersdorf and coworkers (2009) doubt that it is correct to transfer the model of microalgal EPS production (Underwood and Smith, 1998) and the role of EPS from marine to freshwater habitats or even to the endolithic habitat.

In aquatic environments heterotrophic bacteria degrade and modify microalgal EPS (van Duyl et al. 1999; Goto et al., 2001). While microalgae secrete mainly carbohydrates as EPS, bacteria release a higher proportion of proteins (Flemming and Wingender, 2001a; Hoagland et al., 1993; Underwood et al., 2004), an important nutritional source for benthic invertebrates.

In the terrestrial environment, EPS have the additional function to store water and nutrients. Further they protect against various stresses, especially during and after desiccation (Tamaru et al. 2005). The important role of EPS in "subaerial biofilms", microbial communities that live on solid mineral surfaces exposed to the atmosphere, has been reviewed e.g. by Gorbushina (2007). Microorganisms can even survive outer space conditions in a dry state, probably with the help of EPS as lichens show an improved resistance to UV irradiation due to their cortex (Cockell et al., 2007; Sancho et al., 2007; Olsson-Francis et al., 2009). Denuding of horizontal sandstone surfaces in Arizona indicates that algal recolonization occurs at 2 to 3 cm per year. Approximately 20 cm^2 of colonized sandstone surface was removed to a depth of 1 cm by chisel and randomly sampled every 3 month for evidence of colonization. Recolonization began at the edges and moved in toward the center. Denuded vertical surfaces were recolonized somewhat faster and primarily from the upper edge with 4 to 5 cm per year (Bell, 1993). Temporarily endolithic organisms remain in a dry state, but when moistened, they rapidly grow and reproduce. Bell (1993) writes that in pure culture, *Chroococcidiopsis* is encased within a gelatinous sheath that, upon rewetting, swells rapidly and sometimes causes single cells

to be squeezed out. In cryptoendolithic habitats, single cells or small aggregates could be conveyed via capillary water to new locations within the rock. Most of the green microorganisms from cryptoendolithic habitats respond to rewetting with a rapid production of motile stages that use capillary water to extend their area of colonization. Most of the organisms identified from the microhabitats in hot semiarid lands and deserts seem to be widely distributed in soil and freshwater of temperate regions as well (Bell 1993). They may have developed a special adaptation to life in such harsh environments or they may just be “drought-tolerators”. Apparently they utilize an “on-off” metabolism similar to that shown by some soil organisms (Brock, 1975). Certain soil algae withstand long periods of drought (70 years, Bristol, 1919; 69 years, Parker et al., 1969), while others withstand extreme temperatures (up to 160℃, Trainor, 1962, 1983; Setchell, 1903; Schlichting, 1974). When desiccation occurs prior to the temperature increase, heat tolerance is more pronounced than in the hydrated state (Vogel 1955). This is likely the case in the cryptoendolithic habitat. Potts and Friedmann (1981) tested the effects of water stress on hot desert isolates of *Chroococcidiopsis* and *Chroococcus*. They concluded that these organisms are not specially adapted to growth and photosynthesis at low water potentials but rather they are extremely tolerant towards drying. The initiation of photosynthesis upon rewetting is very rapid and similar to that displayed by desert lichens (Kappen et al., 1980). Desiccation tolerance of prokaryotes has been reviewed by Potts (1994). Further, EPS acts as a very good conservation matrix to keep the original structure e. g. in a fossilized biofilm (Tomescu et al. 2008).

Porous rock offers a large inner surface for attachment of microorganisms and allows the phenomenon described by Diels (1914) of the accumulation of phototrophic microorganisms in a small greenish layer a few mm below the rock surface. This colored band is visible by the naked eye when the rock is opened mechanically (Fig.5).

In the past decades many sites with endolithic organisms were detected and characterized, all still based on classical identification (Table 2).

Sites of endolithic colonization cover rock formations in Scotland, in the central Alps, in the Dinaric Alps, and in Arizona, deserts in the Negev, the Death Valley, Central Asia, Mexico, Arctic and Antarctica, soils in Africa and Australia and specific sites in North and South America such as the Niagara Escarpment in southern Ontario, Canada, and the McMurdo Dry Valleys in the Ross Desert in the Antarctica, epi- and endoliths being there the only living vegetation.

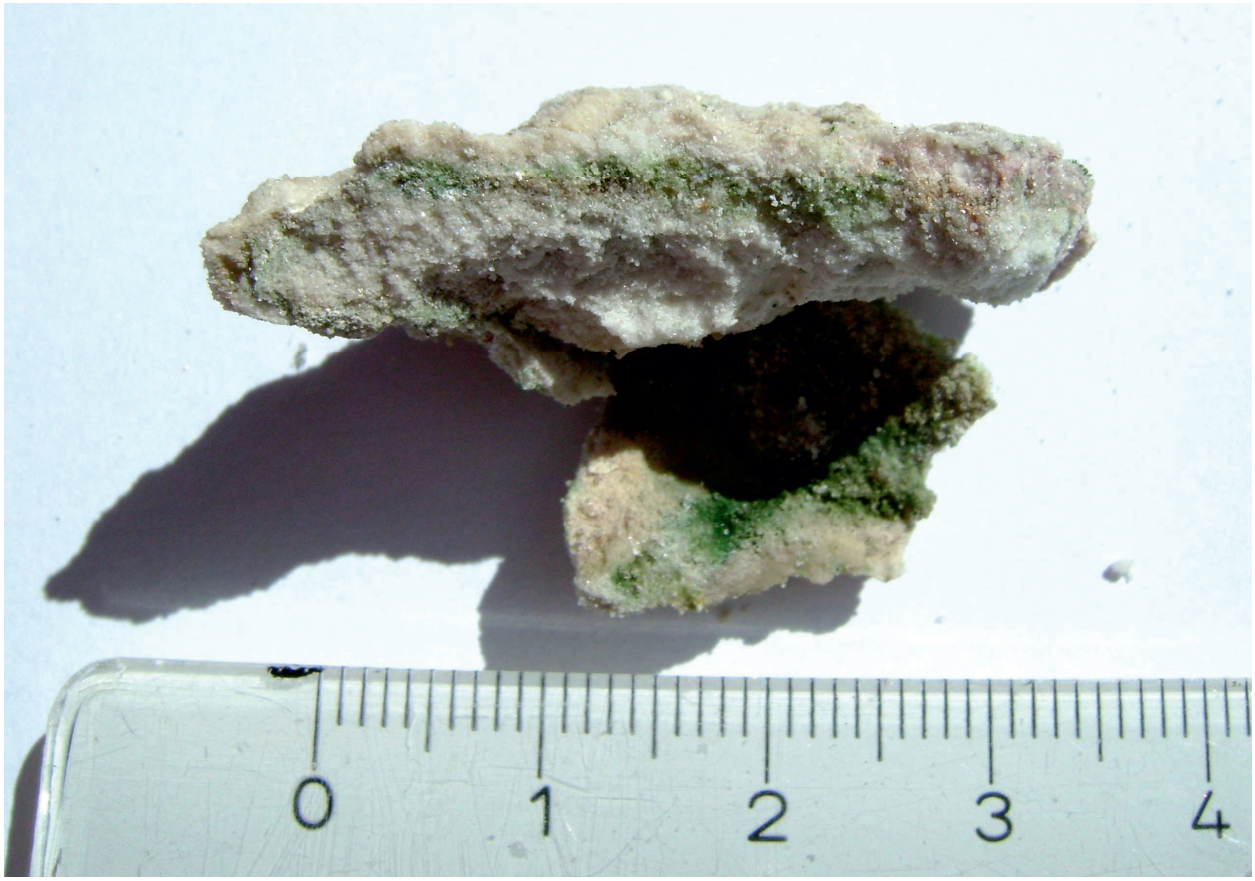

Fig. 5: Opened dolomite rock with endolithic green phototrophs.

Molecular biology has developed new sensitive techniques for microbial ecology which allow to determine the species composition without the need of culturing the organisms. More and more sites with endolithic colonization are being investigated by molecular techniques such as sequencing the small subunit of the ribosomal RNA gene (SSU rRNA gene) and other genes, or using phospholipid or fatty acid markers. As the new SSU rRNA sequences are deposited constantly in the worldwide data base (GenBank, EMBL Nucleotide Database, and DNA Data Bank of Japan), we find to date about 36 submissions of one or more sequences that come from endolithic habitats (Table 3). The molecular work on endoliths covers sites in hot and cold deserts, in high mountain regions, at sea coasts from the arctic to the tropic climate zones, on buildings, and from the sub sea floor. The colonized substrate varies broadly, comprising limestone, sandstone, dolomite, gypsum, hyperarid soil sulfates, travertine (gypsum / limestone), intertidal carbonates, tuff, basalt, siliceous rocks, granodiorite rock, and granite.

Garcia-Pichel et al. (2003) estimated the mass of cyanobacteria worldwide in arid land soil crusts as the astonishing number of 54×10^{12} g carbon, of which 14×10^{12} g carbon of biomass would come from arid land endolithic communities, equal to 4.7% of the total global cyanobacterial biomass.

Table 2: Non-molecular studies on endolithic prokaryotes

Reference	Location	Latitude / longitude	Rock type	Described organisms
Agardh CA (1824) Systema algarum, Lundae: *Literis Berlingianis*				
Kützing FT (1845) Phycologia germanica Deutschlands Algen in bündigen Beschreibungen 1-340. Nordhausen: W. Köhne				
Nägeli C (1849) Gattungen einzelliger Algen, Sonderdruck aus *Neue Denkschr Allg Schweiz Ges Naturwiss*, **10**:1-139				
Bachmann E (1890) Die Beziehung der Kalkflechten zu ihrem Substrat. *Ber Dtsch Bot Ges* **8:**141–145				
Brand F (1900) Der Formenkreis von *Gloeocapsa alpina* Näg. *Botanisches Centralblatt* LXXXIII, **7/8**:224-236; **10**:305-313				*Gloeocapsa alpina* Näg.
Oettli M (1905) Beiträge zur Ökologie der Felsflora. Untersuchungen aus dem Churfirsten- und Sentisgebiet. In: Schroeter C (ed.) Botanische Exkursionen und pflanzengeographische Studien in der Schweiz. 3.Heft.Zürich, Albert Raustein	Churfirsten		limestone	chlorophyllgrüner Fleck (p. 28), Tintenstriche (p. 38) Otto Jaag (1945) comments (p. 420): the green spot is not a common appearance and it is not algae but endolithic lichens
Fritsch FE (1907) The subaerial and freshwater algal flora of the tropics. A phytogeographical and ecological study. *Ann Bot* **21:**235–275				
Schroeter C (1908) Das Pflanzenleben der Alpen. Eine Schilderung der Hochgebirgsflora, Zürich, Albert Raustein (p. 558 - 559)	Hochgebirge kalkige Scheienfluh in Rhaetikon bläulich wegen *Gloeocapsa*		Kalk oder Schiefer feuchte Gneisfelsen in den Schöllenen (C.Cramer)	Salpeterbakterien oder Nitromonaden „Tintenstriche“ Spaltalgen *Gloeocapsa*, *Stigonema*,
Diels FLE (1914) Die Algen-Vegetation der Südtiroler Dolomitriffe. Ein Beitrag zur Ökologie der Lithophyten. *Ber Dtsch Bot Ges* **32**:502–526	Dolomite cliffs, Schlern, Weislahntal, Italian Alps, 1280 m a.s.l.	46°31’N 11°34’E	Dolomite	*Gloeocapsa punctata, Aphanothece, Trentepohlia aurea, Lyngbya* cf. *foveolarum*, *Nostoc* sp. (*Scytonema*?)
Bristol BM (1919) On the retention of vitality by algae from old stored soils *New Phytologist* **18**:92-107	agricultural soil	England, Harpenden, Rothamsted Experimental Station / Luton, Barnfield	soil	some of the algae survived at least 70 years (*Nostoc muscorum* and *Plectonema battersii*) other successful ones: *Chlorococcum humicola, Anabaena oscillarioides, Stichococcus bacillaris*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Blöchliger G (1931) Mikrobiologische Untersuchungen an verwitternden Schrattenkalkfelsen, ETH Diss., Frischknecht & Lüscher, Zürich	Churfirsten, Kalkfelsen			
Geitler L (1932) Cyanophyceae. *In*: „Dr. Rabenhorst's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz" Vol 14,. Akad. Verlagsges., Leipzig. pp 1-1196				"monumental book of Myxophyceae" Gerrath et al. (2000): "All of these genera were recorded previously from soils or in subaerial surface habitats (Geitler 1932)."
Jaag O (1945) Untersuchungen über die Vegetation und Biologie der Algen des nackten Gesteins in den Alpen, im Jura und im schweizerischen Mittelland. *Beitr Kryptogamenflora Schweiz* **9**:1–560	Algae of the high mountains and the Prealps, 250 to 3200 m a.s.l. (4000 m a.s.l.)	region of Säntis, south slope of Churfirsten, around Arosa, Near Bärschis SG, Bürgenstock,. Thierfehd, Lowerzersee, Iberger Egg, Reichenbachfall 45°58'-47°13'N 6°-10°20'E	on limestone, dolomite, quartz, granite, gneiss, serpentine, amphibolite, biotite, nagelfluh, molasse sandstone; from acidic to alkaline (pH 5.1-7.5), from hard to soft,	*Gloeocapsa sanguinea, G. demochroa, G. Kützingiana, Tolypothrix byssoidea, Calothrix parietina, Trentepohlia aurea, Schizothrix epilithica, Scytonema myochrous, Chroococcus turgidus, Sacconema rupestris (Rivularia), Aphanocapsa montana, Nostoc microscopicum, Gloeothece pallida, Synechococcus aeruginosus, Stigonema minutum, Plectonema gracillimum*
Webley DM, Hendersen MEK, Taylor IF (1963) The microbiology of rocks and weathered stones. *European J Soil Sci* **14:** 102–112	Scotland, UK: Clachnaben, Ben Newe, Bennachie, Craig-na-Slice, Birk Hills, Craggan, Tillycorthie, Myreside, Beauty Hill, Portsoy, Pitcaple		granite, diorite rock, quartzose gneiss, granitic gneiss, basic gneiss, amphibolite-chlorite stone, disintegrating serpentine stone, olivine-norite stone	Bacteria, actinomycetes, and fungi in the interior of porous weathered stones, but not in unweathered stones,described as short rods, spore formers, cocci, pleomorphic, and mycelial organisms. A high proportion of the organisms isolated were able to render silicates soluble when tested in pure culture in the laboratory.
Starmach K (1966) Cyanophyta-Sinice, Glaucophyta-Glaukofity. In: Flora Slodkowodna Polski (K Starmach editor) Vol 2 pp 1-808. Polska Akademia Nauk, Panstwowe Wydawnictwo Naukowe, Warszawa.				
Friedmann EI, Lipkin V, Ocampo-Paus R (1967) Desert algae of the Negev (Israel). *Phycologia* **6**:185-200	hot Negev desert, Israel		limestone, dolomite flint stone, plutonic rock	Chlorosphaerales, filamentous blue-green algae coccoid blue-greeen algae

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Golubic, S. (1967) Algenvegetation der Felsen. Eine ökologische Algenstudie im dinarischen Karstgebiet. *In*: Elster HJ, Ohle W (eds) *Die Binnengewässer*, Vol. 23. Schweizerbart'sche-Verlagsbuchhandlung, Stuttgart, pp. 1–183	mountain chain of the Dinaric Alps, Slovenia, Croatia, Bosnia-Herzegovina, Montenegro	42°25' to 45°40' N and 13°30' to 18°30'E	limestone (Jurassic, Cretaceous, Tertiary) frequently dolomite	**Cyanophyta**: *Aphanocapsa, Aphanothece, Gloeocapsa, Gloeothece, Chroococcus, Merismopedia, Entophysalis, Pseudocobyrsa, Chlorogloea, Hydrococcus, Xenococcus, Scopulonema, Hyella, Chamesiphon, Clastidium, Stigonema, Hapalosiphon, Scytonema, Tolypothrix, Homoeothrix, Calothrix, Dichothrix, Rivularia, Nostoc, Microcoleus, Hydrocoleum, Schizothrix, Oscillatoria, Phormidium, Lyngbya, Symploca* **Rhodophyta:** *Bangia, Batrachospermum, Pseudochantransia* **Chlorophyta**: *Gloeocystis, Chlorella, Codcomyxa, Stichococcus, Chaetophora, Pleurococcus, Gongrosira, Trentepohlia, Cladophora.* **Conjugatophyta**: *Oocardium* **Xanthophyta**: *Vaucheria*
Friedmann EI (1971) Light and scanning electron microscopy of the endolithic desert algal habitat. *Phycologia* **10**: 411-428	Death Valley, California, USA and Negev Desert, Israel		fossiliferous and crystalline limestone, sandstone	Chroococcales (all *Gloeocapsa*?) Microscopic examination shows that the algae may be accompanied by bacteria (p.412)
Friedmann EI (1972) Ecology of lithophytic algal habitats in Middle Eastern and North American deserts. *In*: Rodin LE (ed.) Ecophysiological Foundation of Ecosystems Productivity in Arid Zones. Nauka, USSR Acad. Sci. Leningrad, pp. 182-185				Mini review of the diversity of desert microalgae
Friedmann EI, Galun M (1974) Desert algae, lichens, and fungi. *In*: Brown GW (ed.) Desert Biology, Vol. II. Academic Press, New York, pp. 163-203	Deserts: Negev, Death Valley (endolithic) Central Asia Desert, Arctic Desert, Negev, SW US and Mexico	water source: dew, capillary forces keep it, light intensity important factor, Temp: high reflectance because of light color	sandstone, crystalline limestone, amorphous limestone	*Gloeocapsa (Chroococcales), Calothrix desertica, Schizothrix atacamensis, Friedmannia israeliensis*. Genera of hot deserts and Antarctic with xerophilic, mesophilic, and perhaps hydrophilic organisms according to Cameron (1966, 1969): *Protococcus, Protosiphon, Palmogloea, Chlorella vulgaris, Stichococcus subtiilis, Schizothrix, Microcoleus, Nostoc, Scytonema, Porphyrosiphon, Symploca, Oscillatoria, Anacystis thermalis, Anacystis marina, Coccochloris,*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Friedmann EI, Ocampo-Friedmann R (1976) Endolithic blue-green algae in the dry valleys: primary producers in the Antarctic desert ecosystem. *Science* **193:**1247–1249	Victoria Land, Antarctic cold Ross desert / Dry Valleys / ice free desert of Antarctica		sandstone	
Friedmann EI (1977). Microorganisms in Antarctic desert rocks from dry valleys and Dufek Massif. *Antarc. J U.S.* **12**:26-29	Antarctic cold Ross desert / Dry Valleys			
Berner T, Evanari M (1978) The influence of temperature and light penetration on the abundance of the hypolithic algae in the Negev Desert of Israel. *Oecologia* **33**: 255 –260	Negev Desert, Israel	31°N, 35°E	Flint	
Friedmann EI (1978) Melting snow in the dry valleys is a source of water for endolithic microorganisms. *Antarct. J. U.S.* **13**:162-163				
Broady PA (1979) The terrestrial algae of Signy Island, South Orkney Islands. *Br Antarct Surv Sci Rep* **98**:1-117	South Orkney Islands, maritime Antarctica			
Friedmann EI, Kibler AP (1980) Nitrogen economy of endolithic microbial communities in hot and cold deserts. *Microb Ecol* **6**:95-108	North and South America, the Middle East, South Africa, Antarctica		mostly sandstone, few granite, soil, limestone, or breccia	N fixation seems to occur only exceptionally, lichen, blue-greens, *Chroococcidiopsis* is known to fix nitrogen under anaerobic conditions
Friedmann EI (1980) Endolithic microbial life in hot and cold deserts. *Origin Life* **10**:223-235	Negev and Dry Valleys		sandstone	lichens with phycobiot *Trebouxia*,
Broady PA (1981) The ecology of chasmolithic algae at coastal locations of Antarctica. *Phycologia* **20**:259-272	near two Australian research stations in Antarctica (costal locations)	67°26'S 62°53'E and 68°35'S, 77°58'E		*Chroococcidiopsis, Myxosarcina, Gloeotheca, Plectonema, Lyngbya, Calothrix Trochiscia, Chlorella, Stichococcus, Urospora, Desmococcus, Prasiococcus calcarius, Prasiola crispa,* *Fragilaria* (a Bacillariophyte)
Friedmann EI (1982) Endolithic microorganisms in the Antarctic cold desert. *Science* **215**:1045-1053	Victoria Land, Antarctic cold Ross desert / Dry Valleys	exempli gratia: 77°52'S 160°39'E, 77°35'S 160°38'E	sandstone	Trebouxia sp.,Heterococcus sp. (Xanthophyceae)
Danin A, Gerson R, Garty J (1983) Weathering patterns on hard limestone and dolomite by endolithic lichens and cyanobacteria: supporting evidence for eolian contribution to Terra Rossa soil. *Soil Sci* **136:**213–217	Mediterranean region "Terra Rossa", hilltop near the University Givat Ram	31°46'N 35°12'E	hard carbonate rocks, horizontally bedded hard dolomite of the Weradim Formation	endolithic lichens like *Caloplaca alociza*, cyanobacteria like *Gloeocapsa*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Broady PA (1983) The Antarctic Distribution and Ecology of the Terrestrial, Chlorophytan Alga Prasiococcus calcarius (Boye Petersen) Vischer. *Polar Biology* **1**:211-216	Antarctica		Habitats: chasmolithic / sublithic / epilithic / associated with bones, feathers and dry mummified remains of birds and seals/ soil adjacent to bird nesting areas and seal wallows / raw mineral soils / epiphytic / summer meltstreams.	*Prasiococcus calcarius*
Anagnostidis K., Economou-Amilli A, Roussomoustakaki M (1983) Epilithic and chasmolithic microflora (Cyanophyta, Bacillariophyta) from marbles of the Parthenon (Acropolis-Athens, Greece). *Nova Hedwigia* **38:**227–287				review
Tschermak-Woess E, Friedmann EI (1984) *Hemichloris antarctica*, gen. et sp. nov. (*Chlorococcales, Chlorophyta*), a cryptoendolithic alga from Antarctica. *Phycologia* **23**:443–454				*Hemichloris,Chroococcidiopsis, Gloeocapsa*
Friedmann EI, Ocampo-Friedmann R (1984a) Endolithic microorganisms in extreme dry environments: Analysis of a lithobiotic microbial habitat. *In*: Klug M, Reddy LA (eds) Current Perspectives in Microbial Ecology. Am. Soc. Micro. Washington D.C. pp. 177-185	Review of: Negev, SW US, Mexico, central Asia, Arctic, Antarctic, Namibia, Sinai, central Australia, Atacama,		diverse, dependent rather on color, opacity, porosity, fissures than on chem. composition – a certain minimal density also seems to be necessary (not growing in rhyolite) granite, calcite, quartzite, dolomite, sandstone	crypto- and chasmo- endolithic microorganisms *Chroococcidiopsis, Chroococcus turgidus*, accompanied by some small colorless or orange pigmented nonphotosynthetic bacteria (unidentified rods and cocci) from the Negev *Trebouxia, Pseudotrebouxia, Hemichloris antarctica, Chroococcidiopsis, Gloeocapsa*, from the Antarctic cold desert Dry Valleys The cryptoendolithic lichen community also contains colorless and pigmented nonphotosynthetic bacteria

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Dragovich D (1984) The survival of desert varnish in subsurface positions, western New South Wales, Australia. *Earth Surf Proc Landf* **9:**425–434	arid western New South Wales, Australia		desert varnish	
Friedmann EI, Ocampo-Friedmann R (1984b) The Antarctic cryptoendolithic ecosystem: relevance to exobiology. *Origin Life* **14:** 771–776	Antarctic desert		sandstone	Mini review; surface lichens in particularly sheltered microhabitats, primary producers in the soil seem to be absent, the few indigenous soil microorganisms are heterotrophs, utilizing organic matter carried in by winds.
McKay CP, Friedmann EI (1985) The cryptoendolithic microbial environment in the Antarctic cold desert: temperature variations in nature. *Polar Biol* **4:**19–25	Antarctic cold Ross desert / Dry Valleys	77°29'S 160°57'E	porous, translucent Beacon sandstone colonization from 1-2 mm down to 10 mm and more	lichens, algae, fungi, bacteria colored bands!
Bell RA, Athey PV, Sommerfeld MR (1986) Cryptoendolithic algal communities of the Colorado Plateau. *J Phycol* **22**:429-435	Colorado plateau, northern Arizona, southern Utah, Western New Mexico	36°N 111°W	sandstones, soil, loose sand	*Synechococcus, Chroococcales, Stichococcus, Klebsormidium, Chlorosarcinales Chroococcidiopsis, Gloeocapsa, Gloeothece, Anabaena, Phormidium autumnale, Chlorococcum sphacosum, Chlorococcum arenosum, Myrmecia, Chlorella, Coccomyxa, Oocystis marssoni, Borodinella polytetras, Chlorosarcinopsis aggregate, Fasciculochloris boldii, Friedmannia israeliensis, Tetracystis dissociate, Tetracystis isobilateralis, Klebsormidium sterile, Stichococcus bacillaris*
Critchley AT, Wood J, Horiguchi T, Bruton AG (1987) An ultrastructural insight into a cryptoendolithic community. *Proc Electr Microsc Soc S Afr* **17**: 101-102				
Friedmann, E.I., McKay, C.P., and Nienow, J.A. (1987) The cryptoendolithic microbial environment in the Ross Desert of Antarctica – satellite-transmitted continuous nanoclimate data, 1984 to 1986. *Polar Biol* **7**:273–287	Ross Desert of Antarctica		sandstone	
Bell RA, Sommerfield MR (1987) Algal biomass and primary production within a temperate zone sandstone. *Am J Bot* **74**: 294-297	Colorado Plateau in Arizona		sandstone	biomass calculations, coccoid blue-green and coccoid/sarcinoid green algae; *Chroococcidiopsis*, *Gloeothece*, sarcinoid green algae *Borodinella* and *Fasiculochloris*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Bell RA, Athey PV, Sommerfeld MR (1988) Distribution of endolithic algae on the Colorado Plateau of Northern Arizona. *The southwestern Naturalist* **33**:315-322	Colorado Plateau of Northern Arizona		sandstone	
Friedmann EI, Hua M, Ocampo-Friedmann R (1988) Cryptoendolithic lichen and cyanobacterial communities of the Ross Desert, Antarctica. *Polarforschung* **58**:251-260	McMurdo-Dry Valleys, Ross Desert, Antarctica	e.g. Battleship Promontory 76°55'S 160°55'E	Beacon sandstone	lichen, *Hemichloris*, red *Gloeocapsa*, *Hormatonema-Gloeocapsa*, *Chroococcidiopsis*
Vestal JR (1988) Carbon metabolism of the cryptoendolithic microbiota from the Antarctic desert. *Appl Environ Microbiol* **54:**960–965	McMurdo-Dry Valleys, Ross Desert, Antarctica	Linnaeus Terrace 77°36'S 161°05'E		
Siebert J, Hirsch P (1988) Characterization of selected coccal bacteria isolated from Antarctic rock and soil samples from the McMurdo-Dry Valleys (South-Victoria Land). *Polar Biol* **9**:37-44	McMurdo-Dry Valleys (South-Victoria Land) Antarctica	e.g. Linnaeus Terrace 77°36'S 161°05'E	Beacon sandstone	*Micrococcus roseus, Micrococcus agilis, Deinococcus*, coccoid *Arthrobacter*, coccoid *Brevibacterium*
Nienow, JA, McKay CP, Friedmann EI (1988) The cryptoendolithic microbial environment in the Ross Desert of Antarctica: light in the photosynthetically active region. *Microb Ecol* **16**:271–289	Dry Valleys, continental Antarctica	78°S, 162°E	Beacon sandstone	*Hemichloris,* study on lightregime in the stone
Hoffmann, L. (1989) Algae of terrestrial habitats. *Bot Rev* **55:** 77–105				Review of up to 1988 literature about lithic "algae"
Broady PA (1989) Survey of algae and other terrestrial biota at Edward VII Peninsula, Marie Byrd Land. *Antarctic Science* **1**:215-224	Antarctica, ice free coast region	77°00'-78°30'S 152°-154°W	granites and metasediments	algae, mosses, lichens, *Cyanothece aeruginosa*, *Gloeocapsa* spp., *Oscillatoriaceae, Nostoc* sp., *Pseudococcomyxa simplex, Stichococcus bacillaris*,
Johnston CG, Vestal JR (1989) Distribution of inorganic species in two Antarctic cryptoendolithic microbial communities. *Geomicrobiol J* **7:**137–153	Antarctic Ross Desert, McMurdo Dry Valleys	77°36'S, 161°05'E and 76°55'S, 161°00'E	translucent Beacon sandstone, rock with hardened, iron-oxide-stained siliceous crust	A more chemical study on Antarctic cryptoendolithic microenvironments dominated by cyanobacteria
Palmer RJ, Friedmann EI. 1990. Water relations and photosynthesis in the cryptoendolithic microbial habitat of hot and cold deserts. *Microb. Ecol.* **19**:111–118	Ross Desert of Antarctica and Negev Desert, Israel	77°36'S 161°05'E 29°49'N 34°55'E	Beacon sandstone, Nubian sandstone	*Trebouxia*, *Chroococcidiopsis* sp., and heterotrophic bacteria
Tayler S, May E (1991) The seasonality of heterotrophic bacteria on sandstones of ancient monuments. *Int Biodeter* **28:**49–64	Portchester Castle in southern England	50°50'16"N 1°06'52"W	sandstone of ancient monuments	heterotrophic bacteria, Bacillus, *Micrococcus, Moraxella*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Garcia-Pichel F & Castenholz RW (1991) Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. *J Phycol* **27**: 395-409	Calafell, Catalonia, Spain Coast Range, West of Monroe, Oregon, USA Woods Hole, Massachusetts and Laguna Guerrero Negro, Mexico		on limestone outcrops on rocks in intermittent spring Black leathery mat on sandflats (upper intertidal)	"Untreated field sample inocula placed directly in standard media usually yielded fast-growing, opportunistic species that did not represent the original population." *Chroococcus* sp. (Gloeocapsa?) semi-encrusted *Calothrix parietina* *Lyngbya aestuarii*
Johnston CG, Vestal JR (1991) Photosynthetic carbon incorporation and turnover in Antarctic cryptoendolithic microbial communities: Are they the slowest-growing communities on Earth? *Appl Environ Microbiol* **57:**2308-2311	Ross Desert of Antarctica		Beacon sandstone	lichen and cyanobacteria
Braams, J. (1992) Ecological studies on the fungal microflora inhabiting historic sandstone monuments. PhD Thesis. Oldenburg, Germany: Oldenburg University.				
Bell RA (1993) Minireview: Cryptoendolithic algae of hot semiarid lands and deserts. *JPhycol* **29**:133-139				Review, additional to Bell et al. 1986: *Lyngbya* sp. Agardh
Broady PA & Ingerfeld M (1993) Three new species and a new record of chaetophoracean (*Chlorophyta*) algae from terrestrial habitats in Antarctica. *Europ J Phycology* **28**:25-31	Antarctica, Scott Nunataks, Edward VII Peninsula	77°15'S, 145°12'W	soil, stones, and epilithic crusts	*Coccobotrys mucosus, Dilabifilum prostratum, Desmococcus endolithicus, Desmococcus olivaceus*
Friedmann EI, Kappen L, Meyer MA, Nienow JA. (1993) Long-term productivity in the cryptoendolithic microbial community of the Ross Desert, Antarctica. *Microb. Ecol.* **25**:51–69	Dry Valleys, Antarctica	77°36'S 161°05'E, 1600-1650 m altitude	Beacon sandstone	biomass productivity measurement
Nienow JA, Friedmann EI. (1993) Terrestrial lithophytic (rock) communities. *In* Friedmann EI (ed), *Antarctic Microbiology*, New York,Wiley-Liss, pp. 343–412				Review
Friedmann EI (1993) Antarctic Microbiology. New York, Wiley & Sons. pp. 1-634				

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Garcia-Pichel F, Castenholz RW (1993) Occurrence of UV-absorbing, mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity. *Appl Environ Microbiol* **59:**163–169	-	-	"desert rock" see Garcia-Pichel&Castenholz 1991	Mycosporine amino acid like compounds in Bacteria from various habitats *Synechococcus* sp., *Gloeocapsa* sp., *Gloeothece* sp., *Lyngbya* sp.,*Calothrix* sp., *Scytonema* sp., *Nostocal (Diplocolon* Sp.), *Chlorogloeopsis* sp., *Nostoc* sp., *Oscillatoria* sp. *Cyanothece* sp. *Spirulina* sp.
Gerrath JF, Gerrath JA, Larson DW (1995) A preliminary account of endolithic algae of limestone cliffs of the Niagara Escarpment. *Can J Bot* **73:**788–793	Niagara Escarpment, southern Ontario, Canada	43°30'N -45°19'N, 79°75'W - 81°34'W	dolomitic limestone	dark green layer in porous dolomitic limestone bacteria, fungi, blue-green algae (cyanobacteria), green algae, yellow-green algae, occasionally the protonemata of mosses *Chroococcidiopsis* sp., *Gloeothece palea*, *Nostoc sphaericum*, *Phormidium* sp., *Plectonema* sp., *Synechocystis parvula*, *Synechocystis miniscula* *Chlorella* sp., *Klebsormidium flaccidum*, *Pseudopleurococcus printzii*, *Stichococcus bacillaris, Stichococcus minor, Ulothrix subtilis* *Chloridella neglecta*
Wessels DCJ, Büdel B (1995) Epilithic and cryptoendolithic cyanobacteria of Clarens sandstone cliffs in the Golden Gate Highlands National Park, South Africa. *Bot Acta* **108:**220–226		28°31'S 28°25'E	sandstone	
Siebert J, Hirsch P, Hoffman B, Gliesche CG, Peissl K, Jendrach M (1996) Cryptoendolithic microorganisms from Antarctic sandstone of Linnaeus Terrace (Asgard Range): diversity, properties and interactions. *Biodivers Conserv* **5:**1337–1363	Linnaeus Terrace, Antarctica	77°36'S 161°05'E	sandstone	comparative study *Stichococcus* sp., *Gloeocapsa* sp., *Hemichloris* sp., fungi, yeasts
Weber B,Wessels DCJ, Büdel B (1996) Biology and ecology of cryptoendolithic cyanobacteria of a sandstone outcrop in the northern province of South Africa. *Algol Stud* **83:**565–579	northern province of South Africa		sandstone outcrop	
Pentecost A, Bayari S, Yesertener C (1997) Phototrophic microorganisms of the Pamukkale travertine, Turkey: their distribution n influence on travertine deposition. *Geomicrobiol J* **14:**269–283	Pamukkale travertine, Turkey	37°55'26"N 29°07'23"E	micrite and sparite, needle-fiber calcite	*Lyngbya (Phormidium) laminosum*, 17 species of cyanobacteria, 16 diatoms, and 5 *Chlorophyceae*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Ferris FG and Lowson EA (1997) Ultrastructure and geochemistry of endolithic organisms in limestone of the Niagara escarpment. *Can J Microbiol* **43:**211–219	Niagara escarpment, Canada		limestone	mainly *Gloeocapsa,* filamentous cyanobacteria. Heterotrophic bacteria growing as epiphytes on dead and living cyanobacteria, and in epilithic biofilms on pore space walls. Carbon isotope (^{14}C) measurements indicated that atmospheric carbon dioxide is used by the endolithic cyanobacteria. Whole rock multielement analyses revealed an enrichment of phosphorus, barium, lead, and zincin the endolith zone and magnesium, calcium, iron, and copper were depleted.
Matthes-Sears U, Gerrath JA, Larson DW (1997) Abundance, biomass, and productivity of endolithic and epilithic lower plants on the temperate-zone cliffs of the Niagara escarpment, Canada. *Int. J. Plant Sci.* **158**:451–460	Niagara Escarpment, Canada	44°N 80°W: Grimsby 43°12'N 79°34'W, Milton 43°30'N 79°75'W, Emmett Lake 45°13'N 81°27'W, Flowerpot 45°18'N 81°38'W, Purple Valley 44°50'N 81°04'W	dolomitic limestone	Per m^2 73.0 mg *Chl a*, and 19.8 mg *Chl b*, mostly originating from epilithic algae, cyanobacteria, and lichens, when the surface was removed *Chl a* (11.1 mg/m2) and *Chl b* (4.6 mg/m2) The *Chl a* : *Chl b* ratio averaged 3.5 overall and 2.7 for endoliths. The productivity of the endolithic community is low.
Grondona I, Monte E, Rives V, Vicente MA (1997) Lichenized association between *Septonema tormes* sp. nov., a coccoid cyanobacterium, and a green alga with an unforeseen biopreservation effect of Villamayor sandstone at 'Casa Lis' of Salamanca, Spain. *Mycolog Res* **101:**1489–1495	Central to west of Spain, Salamanca, wall of buildings .	40°57'34"N 5°39'60"W	Villamayor sandstone composed of quartz, feldspars and micas	mutualism between a fungus (*Septonema tormes* sp. nov.), a cyanobacterium (*Cyanothece*-group) and a green alga (*Gloeocystis rupestris*)
Büdel B, Karsten U, Garcia-Pichel F (1997) Ultraviolet-absorbing scytonemin and mycosporine-like amino acid derivatives in exposed, rock-inhabiting cyanobacterial lichens. *Oecologia* **112:**165–172				study about scytonemin and mycosporine-like amino acid derivatives
Gross W, Kuver J, Tischendorf G, Bouchaala N, Busch W (1998) Cryptoendolithic growth of the red alga *Galdieria sulphuraria* in volcanic areas. *Euro J Phycol* **33:**25–31	Volcanic springs, Naples, Italy	41°N, 14°E	Amorphous silica	*Galdieria sulphuraria*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Matthes-Sears U, Gerrath JA, Gerrath JF, Larson DW (1999) Community structure of epilithic and endolithic algae on cliffs of the Niagara Escarpment, Ontario, *Canada J Veg Sci* **10**:587–598	Niagara Escarpment, Canada	44℃N 80℃W	dolomitic limestone	13 taxa of cyanobacte ria and 12 taxa of eukaryotic algae were identified in the endolithic zone
Sun HJ, Friedmann EI (1999) Growth on geological time scales in the Antarctic cryptoendolithic microbial community. *Geomicrobiol J* **16:**193–202	McMurdo Dry Valleys (Ross Desert), Antarctica		Beacon sandstone	The color intensity of the rock surface as indicator of relative age of the crust
Büdel B. (1999) Ecology and diversity of rock-inhabiting cyanobacteria in tropical regions. *Eur. J. Phycol.* **34**:361–370	North Transvaal, South Africa,Puerto Ayacucho, Venezuela,French Guyana,Namib Desert, Namibia		granite and sandstone inselbergs (isolated rock outcrops)	Exposed rock surfaces on different continents and under different climatic conditions are occupied by a cosmopolitan, well-adapted, low-diversity microbial community dominated by cyanobacteria and cyanobacterial lichens. *Amphithrix* sp., *Calothrix* sp.,*Chroococcidiopsis* sp., *Chroococcus* sp., *Dichothrix* sp., *Doliocatella* sp., *Entophysalis* sp.,*Geitleribactron* sp., *Gloeocapsa* sp., *Gloeothece* sp., *Hapalosiphon* sp., *Nostoc* sp., *Plectonema* sp. *Porphyrosiphon* sp., *Rivularia* sp., *Schizothrix* sp. *Scytonema* sp. ,*Starria* sp., *Stigonema* ¯sp., *Symphyonema* sp., *Symploca* sp., *Xenococcus* sp.
Van Thielen N, Garbary DJ. 1999. Life in the rocks: endolithic algae. *In*: Seckbach J (ed) *Enigmatic Microorganisms and Life in Extreme Environments*. Dordrecht, Kluwer, pp. 243–253				Review
Gorbushina AA, Krumbein WE, Palinska KA (1999) Poikilotroph growth patterns in rock inhabiting cyanobacteria. *In*: Peschek GA, Löffelhardt W, Schmetterer G (eds). *The Phototrophic Prokaryotes*. New York, Kluwer Academic Publishers, pp. 657–664				

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Gerrath JF, Gerrath JA, Matthes U, Larson DW (2000) Endolithic algae and cyanobacteria from cliffs of the Niagara Escarpment, Ontario, Canada. *Can J Bot* **78:**807–815		43°12'N - 45°19'N, 79°34'W - 81°38'W	outcrop of Silurian dolomitic lilmestone	*Chlorogloea* sp., *Chroococcidiopsis* sp. *Eucapsis*sp., *Gloeocapsa* sp., *Gloeothece* sp., *Nostoc* sp., *Plectonema* sp., *Schizothrix* sp., *Synechocystis* sp., *Coccobotrys* sp., *Muriella* sp., *Pseudendoclonium* sp., *Scotiella* sp., *Stichococcus* sp., *Trebouxia* sp., *Chloridella* sp., *Ellipsoidion* sp., *Heterococcus* sp.
Smith BJ, Warke PA, Moses CA (2000) Limestone weathering in contemporary arid environments: a case study from southern Tunisia. *Earth Surf Proc Land* **25:**1343–1354	Between Remada and Tatahouine, Tunisia	32°39'N 10°19'E	Jurassic and Cretaceous limestone escarpments	geological study on weathering
Banerjee M, Whitton BA, Wynn-Williams DD (2000) Phosphatase activities of endolithic communities in rocks of the Antarctic Dry Valleys. *Microb Ecol* **39:**80–91	McMurdo Dry Valleys, Antarctica		Beacon Sandstone	*Chroococcidiopsis, Gloeocapsa, Trebouxia,*bacteria, phosphomonoesterase activity
Kidron GJ (2000) Dew moisture regime of endolithic and epilithic lichens inhabiting limestone cobbles and rock outcrops, Negev Highlands, Israel. *Flora* **195**:146–153	Negev Highlands, Israel		limestone cobbles and rock outcrops	
Wynn-Williams DD (2000) Cyanobacteria in deserts – life at the limit? *In:* Whitton BA, Potts M (eds) The Ecology of Cyanobacteria – Their Diversity in Time and Space. Dordrecht, the Netherlands, Kluwer Academic Publishers, p. 341–366.	Hot and cold deserts			Review
Matthes, U., Turner, S.A., and Larson, D.W. (2001) Light attenuation by limestone rock and its constraint on the depth distribution of endolithic algae and cyanobacteria. *Int J Plant Sci* **162**: 263–270	Niagara Escarpment, Canada	44°N, 80°W	Limestone	
Rothschild LJ, Mancinelli RL. (2001) Life in extreme environments. *Nature* **409**:1092–1101				Review
Gorbushina AA (2001) Who is living on bare rock? *Priroda* **9:**37–44.				
Wierzchos J, Ascaso C (2001) Life, decay and fossilization of endolithic microorganisms from the Ross Desert, Antarctica. *Polar Biol* **24:**863–868.	Ross Desert Antarctica		porous sandstone rock	predominant: *Gloeocapsa* red, *Hormathonema-Gloeocapsa, Chroococcidiopsis*

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Hughes KA, Lawley B (2003) A novel Antarctic microbial endolithic community within gypsum crusts. *Environ Microbiol* **5**:555-565	Alexander Island, Antarctic Peninsula	72°S, 68°W	Gypsum	
Costerton JW, Stoodley P (2003) Microbial biofilms: protective niches in ancient and modern microbiology. *In:* Krumbein WE, Paterson DM, Zavarzin GA (eds) Fossil and Recent Biofilms: A Natural History of Life on Earth. Kluwer, Dordrecht, the Netherlands, p. xv–xxi				Biofilms as first colonizers of rock
Banerjee NR, Muehlenbachs K (2003) Tuff life: Bioalteration in volcaniclastic rocks from the Ontong Java plateau. *Geochem Geophys Geosyst* **4**:1037	Ocean, Ontong Java Plateau			microscopic textural, geochemical, isotopic, and biomolecular evidence for microbial alteration of glass shards
Büdel B,Weber B, Kühl M, Pfanz H, Sültemeyer D, Wessels D (2004) Reshaping of sandstone surfaces by cryptoendolithic cyanobacteria: bioalkalization causes chemical weathering in arid landscapes. *Geobiology* **2:**261–268	South African sandstone formations		sandstone	Chemical weathering by cryptoendolithic cyanobacteria, increasing pH to enhance silica solution.
Hoppert M, Flies C, Pohl W, Gunzl B, Schneider J. 2004. Colonization strategies of lithobiontic microorganisms on carbonate rocks. *Environ Geol* **46**:421–428	forelands of the Grosser Gosau glacier and Schneeloch glacier (Dachstein, Upper Austria)	47°28'N 13°36'E	Dachstein limestone, slightly dolomitized parts may occur	First colonizers of the outcropping rock on glaciers were nonphotosynthetic organisms, detected *in situ* and cultured. The fungi were followed by the green algae *Stichococcus bacillaris* and *Monodus unipapilla,* noendolithic cyanobacteria.Epilithic cyanobacteria, predominantly *Gloeothece* sp. on wet rock surfaces.
Parnell J, Lee P, Cockell CS, Osinski GR (2004) Microbial colonization in impactgenerated hydrothermal sulphate deposits, Haughton impact structure, and implications for sulphates on Mars. *Intl J Astrobiol* **3**:247–256		75°24'N, 89°45'W		
Süß J, Engelen B, Cypionka H, Sass H (2004) Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. *FEMS Microbiol Ecol* **51:**109–121		32°46.42'N 19°11.55'E 34°48.79'N 27°17.13'E 34°31.39'N 31°46.40'E		Microbial communities of ancient Mediterranean sapropels, buried dark sediment layers of high organic matter

Table 2 (continued)

Reference	Location	Latitude / longitude	Rock type	Described organisms
Wierzchos J, Sancho LG, Ascaso C (2005) Biomineralization of endolithic microbes in rocks from the McMurdo Dry Valleys of Antarctica: implications for microbial fossil formation and their detection. *Environ Microbiol* **7**:566–575	McMurdo Dry Valleys of Antarctica	77°35'S 163°24'E, 120 m 77°60'S, 161°08'E, 1600 m	sandstone rock	
Crispim CA, Gaylarde CC. 2005. Cyanobacteria and biodeterioration of cultural heritage: a review. *Microb Ecol* **49**:1–9	mainly South America			biodeterioration Review
Horath T, Neu TR, Bachofen R (2006) An endolithic microbial community in dolomite rock in Central Switzerland: characterization by reflection spectroscopy, pigment analyses, scanning electron microscopy, and laser scanning microscopy. *Microb Ecol* **51**:353-364	Piora Valley, Swiss Alps	46°33'N, 8°43'E	Dolomite	Coccal and filamentous cya nobacteria, spectral analysis
Omelon CR, Pollard WH, Ferris FG. (2006) Chemical and ultrastructural characterization of high arctic cryptoendolithic habitats. *Geomicrobiol. J.* **23**:189–200	Canadian high Arctic			cyanobacteria, algae, fungi and heterotrophic bacteria

Table 3: Molecular studies on endolithic organisms based on a search in the Nucleotide Database of NCBI with the keywords “endolithic”, “cryptoendolithic”, or “chasmoendolithic”, resulting in 1514, 359, and 2 entries, respectively, in sum 1875 entries.

Reference	Location	Rock type	Gene	Accession Number(s)
Billi D, Grilli Caiola M, Paolozzi L, Ghelardini P (1998) A method for DNA extraction from the desert cyanobacterium *Chroococcidiopsis* and its application to identification of *ftsZ. Appl Environ Microbiol* **64**:4053–4056	Israel: Negev Desert, Makhtesh Ramon	Nubian sandstone	ftsZ, partial cds	AY618316 *Chroococcidiopsis* sp. CCMEE 29 029
Benardini JN, Sawyer J, Venkateswaran K, Nicholson WL (2003) Spore UV and acceleration resistance of endolithic *Bacillus pumilus* and *Bacillus subtilis* isolates obtained from Sonoran desert basalt: Implications for lithopanspermia. *Astrobiol* **3**:709–717	USA, Arizona, Sonoran desert	basalt	16S rDNA partial	AY260858 *Bacillus subtilis* strain WN696 AY260859 *Bacillus pumilus* strains - AY260863 *Bacillus pumilus* strain WN692 AY260864 *Bacillus pumilus* strain WN691
Billi D, Friedmann IE, Ocampo-Friedmann R (2003) Genetic diversity of Chroococcidiopsis (Cyanobacteria) strains from hot and cold deserts revealed by molecular techniques. Unpublished	Mexico, Sonoran Desert	chasmoendolithic in granite	16S rRNA	AY301002 *Chroococcidiopsis* sp. 71-20 strain coidentity: 71-20 = UO-CCMEE 102
Billi D, FriedmannIE, Ocampo-Friedmann R (2003) Genetic diversity of Chroococcidiopsis (Cyanobacteria) strains from hot and cold deserts revealed by molecular techniques. Unpublished.	Israel, Negev Desert	Nubian sandstone	16S rDNA partial	AY301000 *Chroococcidiopsis* sp. N6904J AY301003 *Chroococcidiopsis* sp. A767-47
de la Torre JR, Goebel BM, Friedmann EI, Pace NR (2003) Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. *Appl Environ Microbiol* **69**:3858-3867	Antarctica, McMurdo Dry Valleys	sandstone	SSU rRNA	AY250846 Uncultured *Caliciaceae* sp -AY250898 actinobacterium clone TLT173
Hughes KA, Lawley B (2003) A novel Antarctic microbial endolithic community within gypsum crusts. *Environ Microbiol* **5**:555-565	Antarctica	gypsum crusts	16S rRNA	AF548567 1450 bp *Sphingomonas* sp. eh2
Kurtz HD Jr (2003) *Spirosoma escalantus* sp. nov. and *Spirosoma navajo* sp. nov. Isolated from a Desert Endolithic Community. Unpublished.	USA, Southeastern Utah, Grand Staircase-Escalante National Monument	sandstone rock	SSU rRNA	AY279976 *Spirosoma navajo* strain HDK663 - AY279978 *Spirosoma navajo* strain HDK660 ATCC: BAA-764™ - AY279982 *Spirosoma linguale* ATCC 23276
Sigler WV, Bachofen R, Zeyer J (2003) Molecular characterization of endolithic cyantobacteria inhabiting exposed dolomite in central Switzerland. *Environ Microbiol* **5**:618-627	Switzerland, Piora Valley	dolomite	16S rRNA	AY153464 cyanobacterium clone 5-41 - AY153448 Uncultured cyanobacterium DGGE band C1

Table 3 (continued)

Reference	Location	Rock type	Gene	Accession Number(s)
Tanaka T, Yan L, Burgess G (2003) *Microbulbifer arenaceous* sp. nov., a new endolithic bacterium isolated from the inside of red sandstone. *Curr Microbiol* **47**:412-416	Scotland, South East Coast	red sandstone	16S rRNA	AJ510266 *Microbulbifer arenaceous*, type strain RSBr-1T. From "Uruguay:Eastern Coast"
Gugger MF, Hoffmann L (2004) Polyphyly of true branching cyanobacteria (*Stigonematales*) *Int J Syst Evol Microbiol* **54**:349-357	South Africa	sandstone in freshwater	16S rRNA	AJ544080 *Nostochopsis lobatus*, strain 92.1
Hirsch P, Mevs U, Kroppenstedt RM, Schumann P, Stackebrandt E (2004) Cryptoendolithic Actinomycetes from antarctic sandstone rock samples: *Micromonospora endolithica* sp. nov. and two isolates related to *Micromonospora coerulea* Jensen 1932. *Syst Appl Microbiol* **27**:166-174.	Antarctica	sandstone	16S rRNA	AJ560637 *Micromonospora* sp. DSM 44396 AJ560636 *Micromonospora* sp. DSM 44397 AJ560635 *Micromonospora endolithica*, type strain DSM 44398T (cryptoendolithic)
Bjelland T, Ekman S. (2005) Fungal diversity in rock beneath a crustose lichen as revealed by molecular markers *Microb Ecol* **49**:598–603	Norway	sandstone	ITS 1, 5.8S rRNA, ITS 2	AY612326 –AY612335 endolithic hyphae
de Los Rios A, Sancho LG, Grube M, Wierzchos J, Ascaso C (2005) Endolithic growth of two *Lecidea* lichens in granite from continental Antarctica detected by molecular and microscopy techniques. *New Phytol* **165**:181-190	Antarctica, Ross Sea Coast, Granite Harbor	granite	18S rDNA, partial; ITS1, 5.8S rDNA, ITS2; 26S rDNA partial	AY667582 – AY667580 AY667583 *Buellia frigida* AY667580 Uncultured *Trebouxia* photobiont of *Lecidea* sp. (ADLR 2004)
Selbmann L, de Hoog GS, Mazzaglia A, Friedmann EI, Onofri S (2005) Fungi at the edge of life: cryptoendolithic black fungi from Antarctic desert *Stud Mycol* **51**:1–32	Antarctica, Northern and Southern Victoria Land, Widowmaker pass and other places	Beacon sandstone	SSU rRNA, (ITS1, 5.8S rRNA, ITS2)	cryptoendolithic black fungi, DQ066713 *Cryomyces antarcticus* strain CBS 116301 = CCFEE 534 – DQ066716 *Friedmanniomyces simplex* strain CBS 116775 DQ028269 *Cryomyces antarcticus* strain CBS 116301 – DQ028272 *Friedmanniomyces endolithicus* strain CCFEE 522
Walker JJ, Spear JR, Pace NR (2005) Geobiology of a microbial endolithic community in the Yellowstone geothermal environment. *Nature* **434**:1011-1014	USA, Yellowstone National Park	rock interior	16S rRNA	AY911495 – AY911423 uncultured “NEC clones”
Chacon-Baca E, Berrendero E, Garcia-Pichel F (2006) Molecular genetic and geologic signatures of endolithic microbial communities in carbonates from Cabo Rojo, Puerto Rico. *Sedimentary Geology* **185**:215-228	Puerto Rico, Cabo Rojo	intertidal carbonates, volcanic rocks, limestones	16S rRNA	DQ380390 Uncultured cyanobacterium DGGE gel band 1B – DQ380408 *Oscillatoriales* cyanobacterium BC006; DQ380395, *Calothrix* sp. BC001; DQ380404, Uncultured cyanobacterium isolate DGGE gel band 7C

Table 3 (continued)

Reference	Location	Rock type	Gene	Accession Number(s)
Fajardo-Cavazos P, Nicholson W (2006) *Bacillus* endospores isolated from granite: Close molecular relationships to globally distributed *Bacillus* spp. from endolithic and extreme environments. *Appl Environ Microbiol* **72**:2856-2863	USA, Arizona, Santa Catalina mountain range near Tucson	granite / basalt at a depth of 3 to 5 cm	SSU rRNA	DQ275185 *Bacillus* sp. WN613 - DQ275174 *Bacillus* sp. WN559
Gaylarde PM, Jungblut A-D, Gaylarde CC, Neilan BA (2006) Endolithic phototrophs from an active geothermal region in New Zealand. *Geomicrobiol J* **23**:579–587	New Zealand, Waimangu Valley and Wai-O-Tapu geothermal region	geothermal siliceous rocks, tuff cliff, Iodine Terraces, silica deposits	16S rRNA	DQ235801, endolithic *Scytonema* sp. CCG1, DQ235802 endolithic *Mastigocladopsis* sp. CCG2; DQ235803 *Nostoc* sp. CCG3, endolithic; DQ235804, *Nostoc* sp. CCG4, endolithic; DQ235805, *Hapalosiphon* sp. CCG5, endolithic; DQ235806, *Hapalosiphon* sp. CCG6 "endolithic", DQ235807 *Phormidium* sp. CCG7 “epilithic"
McNamara CJ, Perry TD, Bearce KA, Hernandez-Duque G, Mitchell R (2006) Epilithic and endolithic bacterial communities in limestone from a Maya archaeological site. *Microb Ecol* **51**:51-64	Mexico, Yucatan, Ek’Balam	limestone, rock interior and rock surface	16S rRNA	AY674870 – AY674841 endolithic clones AY674840 – AY674787 epilithic clones
Norris TB, Castenholz RW (2006) Endolithic photosynthetic communities within ancient and recent travertine deposits in Yellowstone National Park. *FEMS Microbiol Ecol* **57**:470-483	USA, Yellowstone National Park	travertine (gypsum / limestone)	SSU rRNA	AY790835 – AY790874 CCMEE deposits AY790390 – AY790472 chlorophyte, bryophyte, cyanobacteria clones
Yoon HS, Ciniglia C, Wu M, Comeron J, Pinto G, Pollio A, Bhattacharya D (2006) Establishment of endolithic populations of extremophilic Cyanidiales (Rhodophyta). *BMC Evol. Biol*. **6**:78-89	Italy, geothermal rocks; Sasso Pissano, Monte Rotondo		ribulose-1,5-bis-phosphate carboxylase /oxygenase LSU, calmodulin	DQ916745 – DQ916749 *Galdieria sulphuraria* XY rbcL DQ916750 – DQ916753 *Cyanidium caldarium* XY rbcL DQ916754 – DQ916827 *Galdieria sulphuraria* XY CaM (*Eukaryota; Rhodophyta; Bangiophyceae, Cyanidiales*)
Dong H, Rech JA, Jiang H, Sun H, Buck BJ (2007) Endolithic cyanobacteria in soil gypsum: Occurrences in Atacama (Chile), Mojave (United States), and Al-Jafr Basin (Jordan) Deserts. *J. Geophys. Res.* **112**:G02030	Jordan, Al-Jafr Basin Chile, Atacama Desert USA, Mojave Desert	hyperarid soil sulfates	SSU rRNA	EF071489Jordan-culture1 (*Roseomonas* sp.) – EF071495Jordan-culture7 (Uncult. *Halomonas* sp.); EF071496Atacama-contB5 (Uncult. *alpha proteobacterium*)- EF071504Atacama-contB67; EF071505 Atacama-colB1-EF071528 Atacama-colB83; EF071529MojaveB2 (uncult. *Desulfosporosinus* sp.)- EF071542MojaveB71 EF071543JordanB5 – EF071546JordanB19 (uncult. *Sphingobacterium*)

Table 3 (continued)

Reference	Location	Rock type	Gene	Accession Number(s)
de los Rios A, Grube M, Sancho LG, Ascaso C (2007) Ultrastructural and genetic characteristics of endolithic cyanobacterial biofilms colonizing Antarctic granite rocks. *FEMS Microbiol Ecol* **59**:386-395	Antarctica, Ross Sea coast, Granite Harbor	granite	16S rRNA	EF094465, endolithic biofilm tightly (or loosely EF094464) attached to lithic substrate.
Fox CH, Carrino-Kyker SR, Swanson AK (2007) Molecular characterization of epilithic and endolithic intertidal biofilm communities along a temperate tidal height gradient. Unpublished	Canada, Dixon Island, northeast Pacific Ocean, northwest Pacific landmass	"granodiorite rock", granite tiles at the intertidal zone	SSU rRNA	EF452622 – EF452632 Uncultured eukaryotes isolate DGGE bands, EF502005 – EF502019 (EF502005 Uncultured bacterium isolate DGGE band1); Epi- and endolithic biofilms
Mason OU, Stingl U, Wilhelm LJ, Moeseneder MM, Di Meo-Savoie CA, Fisk MR, Giovannoni SJ (2007) The phylogeny of endolithic microbes associated with marine basalts. *Environ Microbiol* **9**:2539-2550	Pacific Ocean	submarine basalt	16S rRNA	EF581296 – EF581286, EF067915 – EF067906, EF067905 – EF067904, EF067903 – EF067901, EF067900 – EF067896, aerobic and anaerobic enrichment cultures innoculated with basalt collected from Brown Bear Seamount or from the Juan de Fuca Ridge
Walker JJ, Pace NR (2007a) Phylogenetic composition of Rocky Mountain endolithic microbial ecosystems. *Appl Environ Microbiol* **73**:3497-3504	USA, Rocky Mountains	sandstone, limestone, granite	16S rRNA (and 18S rRNA?)	EF522777 – EF522703 (SCSS), EF522493 – EF522449 (OCSS), EF522362 – EF522221 (FQSS), EF522702 – EF522642 (SCLS), EF522448 – EF522363 (OCLS), EF522220 – EF522190 (EPLS), EF522641 – EF522494 (SCGR)
Horath T, Marty E, Mueller S, Hanselmann KW (2008) 16S rRNA gene sequences of endolithic bacteria and archaea from Weissenstein (white rock) / Albula pass. Unpublished	Switzerland, Weissenstein, Albula Pass	dolomite	16S rRNA	AB473894 EM_01_BAC –AB473913, AB473914 SM_20_BAC –AB473922
Horath T, Schmid N, Hanselmann K (2008) 16S rDNA sequences from Weissenstein (white rock) / Albula pass. Unpublished	Switzerland, Weissenstein, Albula Pass	dolomite	SSU rRNA	AB374402 – AB374366
Kurtz HD Jr, Davis S, Glaros T, Sannem S (2008) Molecular Diversity within a Cryptoendolithic Microbial Community. Unpublished	USA, Southeastern Utah, Grand Staircase-Escalante National Monument	sandstone formations	SSU rRNA	EU751343 Propionibacterineae bacterium clone CA-E11 – EU751383 *Segetibacter* sp. clone HK05-F11 / EU751312 Acetobacteraceae bacterium clone SD-E05 – EU751342 Betaproteobacterium clone HK05-B08 / EU751384 Chloroflexaceae bacterium clone CA-A01 – EU751603 eukaryote clone HK03-H12

Table 3 (continued)

Reference	Location	Rock type	Gene	Accession Number(s)
Knowles EJ, Castenholz RW (2008) Effect of exogenous extracellular polysaccharides on the desiccation and freezing tolerance of rock-inhabiting phototrophic microorganisms. *FEMS Microbiol Ecol* **66**:261-270	USA, Yellowstone National Park, Painted Pool Terrace	travertine rock	16S rRNA	EU087570 *Nostoc* sp. CCMEE 6160
Mikhailyuk TI, Sluiman HJ, Massalski A, Mudimu O, Demchenko EM, Kondratyuk SY, Friedl T (2008) New streptophyte green algae from terrestrial habitats and an assessment of the genus Interfilum (Klebsormidiophyceae, Streptophyta) *J Phycol* **44**:1586-1603	Antarctica, Marie Bird Land		18S rRNA	EU434026 *Desmococcus endolithicus* strain SAG 25.92 gene
Muggia L, Grube M, Tretiach M (2008) A combined molecular and morphological approach to species delimitation in black-fruited, endolithic *Caloplaca*: high genetic and low morphological diversity. *Mycol Res* **112**:36-49	diverse rocky places in Italy and Austria	limestone	ITS 1 partial; 5.8S rRNA complete, ITS 2 partial	EF095230 - EF095235, Uncultured *Trebouxia* photobiont sequences EF081035 - EF081040 *Caloplaca badioreagens* voucher sequences EF090920 - EF090936 Caloplaca sp. isolates EF093564 - EF093580 Caloplaca sp. isolates
Wong FKY, Lau MCY, Aitchison JC, Pointing SB (2008) Endolithic microbial communities of limestone niches in a high-altitude arid environment. Unpublished	China, Tibet, ,high altitude arid environment	limestone	SSU rRNA	FJ489993 Uncultured archaeon -FJ490055 Uncultured fungus
Banerjee M, Craig Everroad R, Castenholz RW (2009) An unusual cyanobacterium from saline thermal waters with relatives from unexpected habitats. *Extremophiles* **13**:707–716	Iceland	siliceous crust along the upper submerged edge of the lagoon	16S rRNA	EF539879, Iceland , *Leptolyngbya* sp. *2e*
Favero-Longo SE, Borghi A, Tretiach M, Piervittori R (2009) *In vitro* receptivity of carbonate rocks to endolithic lichen-forming aposymbionts *Mycol Res* **113**:1216-1227	Italy, Trieste	limestone	18S rRNA partial, ITS1, 5.8S rRNA, ITS2 partial	EF369524 *Verrucaria marmorea* isolate 805 lichen-forming mycobiont isolated from thalline EF369523 - EF369521
Horath T, Bachofen R (2009) Molecular Characterization of an Endolithic Microbial Community in Dolomite Rock in the Central Alps (Switzerland) *Microb Ecol* **58**:290–306	Switzerland, Piora Valley	dolomite	SSU rRNA	AB257687, AB257667, AB257653

A special subgroup of habitats for endolithic organisms is represented by the stony walls of buildings and monuments. Studies of these habitats mostly focus on the deteriorating action or protection of the stone material by the endolithic microorganisms. Macedo and coworkers (2009) reviewed 32 scientific papers published between 1976 and 2009 on cyanobacteria and chlorophyta that cause deterioration of stone cultural heritage such as outdoor monuments and stone works of art in European countries of the Mediterranean Basin. The artificial environments are classified into six lithotypes: which are also the main substratum types in the natural habitats: marble, limestone, travertine, dolomite, sandstone, and granite. The most widespread and commonly reported taxa on the stone cultural heritage – again similar to what has been detected with the microscope and culturing in the natural habitat – are the cyanobacteria *Gloeocapsa*, *Phormidium*, and *Chroococcus* and the chlorophyta *Chlorella*, *Stichococcus* and *Chlorococcum*. *Cyanobacteria* and *chlorophyta* colonize a wide variety of substrata and this is related primarily to the physical characteristics of the stone surface, microclimate, and environmental conditions and only secondarily to the lithotype (Tiano et al., 1995; Tomaselli et al., 2000; Macedo et al., 2009). The extent of microbial colonization appears to increase as the surface roughness increases (Tomaselli et al., 2000; Morton et al., 1998; Donlan, 2002; Macedo et al., 2009). If the presence of cyanobacterial and algal biofilms on stone monument surfaces can be considered biodeteriogenic, simply because of the aesthetic damage they cause by producing variously colored patinas (Macedo et al. 2009), can be discussed. Not deniable however is the fact, that growing bacteria can develop a certain pressure on their housing thereby causing surface detachment, superficial losses, or increased porosity (Griffin et al., 1991; Saiz-Jimenez, 1999). Better investigated is the release of corrosive acids, such as lactic, oxalic, succinic, acetic, glycolic or pyruvic acid or also the production of carbon dioxide by respiration of aerobic microorganisms. The acids etch and solubilize the stone which leads to its biodeterioration (Danin and Caneva, 1990; Griffin et al., 1991; Caneva et al., 1992; .Wakefield and Jones, 1998; Fernandes, 2006). Still, concerning historical monuments, we can ask ourselves, what did do more damage to the stones and sculptures for example in Rome: two millennia of microbial coating or one century of environmental pollution? Today, the most important threat to historical monuments is still acidic rain (Honegger and Aptroot, 2008). Therefore, one possibility to prevent (bio-) corrosive damage would probably be to keep the object dry. "In general, the conservation approach to the treatment of stone monuments affected by biodeterioration is characterized by extremes. The most common approach can be characterized as a non-intervention approach, and derives from the

perception that other causes of deterioration such as soluble salt migration and freeze-thaw cycling are primary causes of degradation. Biodeterioration is typically seen as only a cosmetic problem: it is noted chiefly as a difference in appearance from unaffected stone. This approach reflects a misunderstanding of the nature of biodeterioration and its synergistic effect on other degradative processes." (Griffin et al. 1991). On the other hand, Grondona and coworkers report about a microbial mat, that, in association with the external crust, avoids a further weathering of the stone because of an unforeseen biopreservation effect due to keeping humidity at constant levels under the crust. This avoids changes in clay swelling and subsequent surface arenization of the sandstone (Grondona et al., 1997).

1.4. Environmental factors and life in the lithosphere

1.4.1. Macronutrients

Microbes grow in almost any environment in the presence of liquid water if their nutrient and energy requirements are met and the environmental conditions can be tolerated. Almost all rock formations have a capacity to support at least limited microbial growth due to the presence of carbon sources, electron donors and acceptors, and macronutrients (C, H, O, N, P, S, K, Cl, Na, Ca, Mg, (Madigan et al. 2002). Among the many chemical factors, nutrients especially limit microbial life. The main growth limiting factor for microorganisms in soil is the availability of nutrients, carbon, nitrogen and phosphorus (Little and Wagner 1996; Aldén et al., 2001). Often sulfur and magnesium are included as limiting factors (Wilkinson, 1958; Mandeva et al., 1981; Mittelman, 1985; Egli, 1991; Fagerbakke et al., 1996, Rhee et al., 1998). Sulfur is often overlooked as a limiting factor as it is available in most soils in sufficient quantities. Deep groundwaters and sediments are often limited in organic carbon and represent an extreme oligotrophic habitat for microorganisms. Direct microscopic counts or basal respiration rates were positively correlated with total organic carbon (Kieft et al. 1995). In dolomite- and lime-stone, carbon is available as CO_2 in the gas phase and as CO_3^{2-} in pore water. These are assimilated mainly by phototrophic microorganisms. Later, carbon becomes available as total organic carbon (TOC) from compounds excreted by the phototrophs or by decaying biomass. Oxygen is available from the atmosphere by diffusion. Nitrogen may be present as nitrate, ammonia or organic nitrogen and further also origin from cyanobacteria through nitrogen fixation or from atmospheric deposition. Odintsova (1941) reported nitrogen-fixing activity of chasmoendolithic (blue-green?) algae in the cold desert of Western Pamir. In zones

between the margin of the permanent snow and 4000 meters above sea level, certain rock types contain considerable amounts of nitrates and harbor in numerous cracks of the friable rock the small cyanobacterium *Gloeocapsa minor* (Kütz.) Hollerb. It has been shown to fix atmospheric nitrogen under laboratory conditions. Tchan and Beadle (1955) described nitrogen-fixing cyanobacteria from "endadaphic" and hypolithic habitats in Australia. In contrast, Friedmann and Kibler (1980) tested hot and cold desert rocks for nitrogen-fixing endoliths with negative results, but found nitrate levels of 0.1 – 25.9 µg g^{-1} and ammonium levels of <2 µg g^{-1}. The detrital layer may also provide soluble nutrients as water percolates through it (Bell, 1993). The amount of total nitrogen in the dolomite rock of the Piora Valley has been measured to be about 200 µg g^{-1} (Sigler et al. 2003), which is not limiting for the amount of biomass being produced in this habitat. Phosphorus can be picked up from inorganic phosphates (PO_4^{2-}), extracellular DNA, phospholipids or polyphosphates, Sulfur from sulfate (SO_4^{2-}), sulfide (H_2S), elemental sulfur S^0, or organic sulfur compounds. Sigler et al. (2003) found both, phosphorus (P <20 µg g^{-1}) and sulfur (S = 156 µg g^{-1}) in the Piora Dolomite, obviously sufficient for the colonization of the rock. To day the atmospheric deposition is probably the main source of nitrogen in industrial countries.

1.4.2. Light

Light is the second most important growth factor in any endolithic environment. The phototrophic organisms produce biomass and supply the heterotrophic population with organic substrates from exudates or decaying cells. Chemolithoautotrophic microorganisms are probably only present in low numbers, as potential electron donors are scarce in the dolomite. It is fascinating how little of sun power will drive photosynthesis inside the stone. As many endolithic habitats are at higher altitude or in regions lacking shading vegetation, the percentage of the inhibitory UV-radiation is high. In alpine lakes UV penetrated down to 2 m depth and diminished photosynthetic activity (Pasini and Schanz, 1998). Light intensity is an important factor for the vertical distribution of microorganisms in the rock; the upper and generally sharp borderline of the endolithic phototrophic zone may be determined by the maximum tolerable level of irradiation, while the lower often diffuse level probably indicates the minimum threshold of useful light (Friedmann and Galun, 1974). Light penetration is affected by physical properties, such as rock color, mineralogy, and structure (Walker and Pace, 2007b). The dolomitic stone has an astonishing transmissibility for light. Diels found for the Schlern Dolomite about 0.03 %

of surface light intensity in 4 mm depth (Table 4). For Piora dolomite similar data are given by Horath et al. (2006).

Table 4: Light transmission of Schlern-Dolomite for different wave lengths (Diels, 1914)

stone thickness	1 mm	2 mm	3 mm	4 mm
middle red [a]	13 %	2 %	0.2 %	0.03 %
D-line [b]	9 %	0.8 %	0.1 %	0.01 %
blue-green [c]	6 %	0.4 %	0.02 %	0.001 %
violet [d]	6 %	0.3 %	0.01 %	<0.001 %

[a] estimated middle wavelength for red light: 650 nm

[b] Na D-line emission is at about 589 nm (see also “sodium vapor lamp” in Wikipedia)

[c] estimated middle wavelength for blue-green light: about 510 nm

[d] estimated wavelength for violet light: about 380 – 420 nm

As illustrated before (Fig. 5), phototrophic endoliths form a distinct layer within the rock. Already Diels (1914) described a border of growth against both the outside and the inside of the rock. At about 6 to 8 mm below the surface the density of stained organisms fades out suggesting that light intensity might be the limiting factor for growth. A similar experiment with the dolomite from the Piora Valley with white light showed a drop of light intensity to 12% to 18% of the surface intensity after 1 mm and a drop to about 2% intensity at 3.5 mm depth in homogenous Piora Dolomite (Horath et al., 2006; Bachofen et al. 2006). These data suggest that the organisms do not get enough light for photosynthesis in regions deeper than 8 mm below the surface. Nevertheless, by confocal laser scanning microscopy, phototrophic organisms were detected down to 20 mm (Horath et al. 2006); but it is not known whether they are still active. However, phototrophic green sulfur bacteria have recently been reported at 100 m depth in the Black Sea (Manske et al. 2005) and at 2391 m depth in the East Pacific Rise (TY black smoker, 330°C, 9°49.63’ N, 104°17.37’ W) (Beatty et al. 2005). These organisms must be able to live at very low light intensities. The average energy sufficient to drive photosynthesis in these habitats was measured to be about 2.2 to 0.75 nmol photons m^{-2} s^{-1} for the Black Sea (Manske et al., 2005) and about 3.6 pmol photons m^{-2} s^{-1} at 700 nm to 800 nm wavelength for the site at the East Pacific Rise (White et al., 2000). Knowing the intensity of sun light which reaches the surface, we can calculate the amount of photons at 4 mm depth of the dolomite rock. Hoel and Solhaug (1998) estimated the natural summer sunlight to be about 1000 µmol photons m^{-2} s^{-1}. Bell (1993) measured the surface photosynthetic active radiation on the Colorado Plateau in the United States to reach levels of 2200 µmol photons m^{-2} s^{-1} in

summer and 500 µmol photons m^{-2} s^{-1} in winter. On this basis, 1% of sunshine would be at least 5 µmol photons m^{-2} s^{-1} and thus at least 1000 times more than the irradiance for the organisms dwelling in 100m depth in the Black Sea and about one million times more than at the TY black smoker site. These numbers make it plausible that the organisms situated at 20 mm depth may still get enough light to drive photosynthesis. While Jaag (1945) suggested that available water is limiting growth towards the inside, we hold light as the more limited source for growth at this site. Table 5 gives an overview on the penetration of light through different rock types (Hughes and Lawley, 2003).

Table 5: Penetration of Light through rocks colonized by endolithic phototrophs.[a]

Reference	**Matthes et al. (2001)**	**Gross et al. (1998)**	**Berner & Evanari (1978)**	**Hughes & Lawley (2003)**	**Nienow et al. (1988)**	**Diels (1914)**	**Horath et al. (2006)**
Location	Niagara Escarpment, Canada	Volcanic springs, Naples, Italy	Negev Desert, Israel	Alexander Island, Antarctic Peninsula	Dry Valleys, continental Antarctica	Dolomite cliffs, Italian Alps	Piora Valley, Swiss Alps
Latitude / longitude	44°N, 80°W	41°N, 14°E	31°N, 35°E	72°S, 68°W	78°S, 162°E	46°31'N 11°34'E	46°33'N, 8°43'E
Rock type	Limestone	Amorphous silica	Flint	Gypsum	Sandstone	Dolomite	Dolomite
Approx. depth of 1% transmission	0.5 – 1 mm	2 mm [b] (0.6% transmission at 500 nm)	5 – 15 mm [b,d] (400 – 750 nm)	1.2 mm [b] (400 – 600 nm)	3 mm [b,c]	2 mm [b]	4.5 mm [e]
Approx. depth of 0.01% transmission	2.1 – 4.5 mm	not determined	10 – 40 mm [b,d] (400 – 750 nm)	2.5 mm [b] (400 – 600 nm)	4 – 6.5 mm [b,c]	3 – 4 mm [b]	more than 4 mm [e]
Depth of endolithic phototrophs	1.1 - 3.5 mm	1 – 5 mm	< 40 mm (hypolithic algae)	0.5 – 3 mm	0.4 – 4 mm	1 – 8 mm	1 – 8 mm

[a] Light penetration varied with rock pigmentation and the presence of endolithic organisms.

[b] Transmission declined with decreasing wavelength.

[c] Calculated from model.

[d] Transmission varied with flint colour and transparency.

[e] Extrapolated from graph

1.4.3. Water

Cellular activity is reduced or inhibited at dry conditions. Water available from rain or dew, or the condensation at the rock surface is imbibed in the porous rock by capillary force (Friedmann and Galun 1974) and produces a microclimate in a stone in the desert quite different from the hot desert macroclimate. The porous rock with its imperforate surface layer represents a system suitable to trap and to retain moisture and to ensure balanced temperature conditions. By a slowly evaporating water reservoir the porous rock maintains

a high level of moisture also during daytime. Bell (1986) measured relative humidity within Sandstone of the Colorado Plateau at or near the algal zone. Regardless of season or rock color, the internal relative humidity dropped never below 60% and stayed usually above 80%, probably adequate to support metabolism in some taxa. The ability to utilize water vapor varies widely for green algal and cyanobacterial phycobionts (Lange et al., 1986). Cryptoendolithic cyanobacteria from Negev Desert sandstones photosynthesize only at high water potentials of >6.9 MPa, 90% relative humidity at 20℃ (Potts and Friedmann, 1981; Palmer and Friedmann 1990). Furthermore the exopolymeric substances (EPS) act like a sponge when water is present and later as a hard protecting shield against water loss. This makes the endolithic biofilm well protected against longer periods of drought.

1.4.4. Temperature

An important physical factor limiting life is the temperature. At most sites of endolithic populations the daily temperature amplitude is great with heating up during the day and often frost in the night. The dolomite surface in the Piora valley rose on midday end of August to 35℃ at an air temperature of 15℃. Howev er, the low heat conductivity of porous rock, together with a high reflectance of the white rock surface prevents a rapid rise of temperature at increasing **in**coming **sol**ar radi**ation** (insolation) and evaporation from the rock further reduces extreme temperature fluctuations inside the rock (Friedmann and Galun 1974). In holes and cavities not exposed to direct light the temperature of the rock surface stays within close limits at ambient air temperature (see also Fig.6). The rock also acts as heat reservoir and thereby buffers the extreme heat peaks. Diels observed a lagging behind of the heat peaks by one or two days of the inner rock temperature compared with the air directly above the stone surface (Diels, 1914). In hotter climatic zones, sandstones appear to act as thermal collectors. They conduct heat into the matrix of the rock; therefore, endolithic organisms endure higher temperatures than they would experience on the surface (Friedmann 1980, Bell 1986, Bell 1993). In the Antarctic, such a phenomenon is helpful since temperatures there rarely rise over -15℃ and most precipitation falls as snow. Antarctic communities rely on solar radiation to raise temperatures towards 0℃ for photosynthesis and to melt snow (Friedmann 1982, Nienow and Friedmann 1983). Many isolates from the Antarctica are psychrophiles with optimal growth temperatures between 0℃ to 20℃ (Siebert et al., 1996).

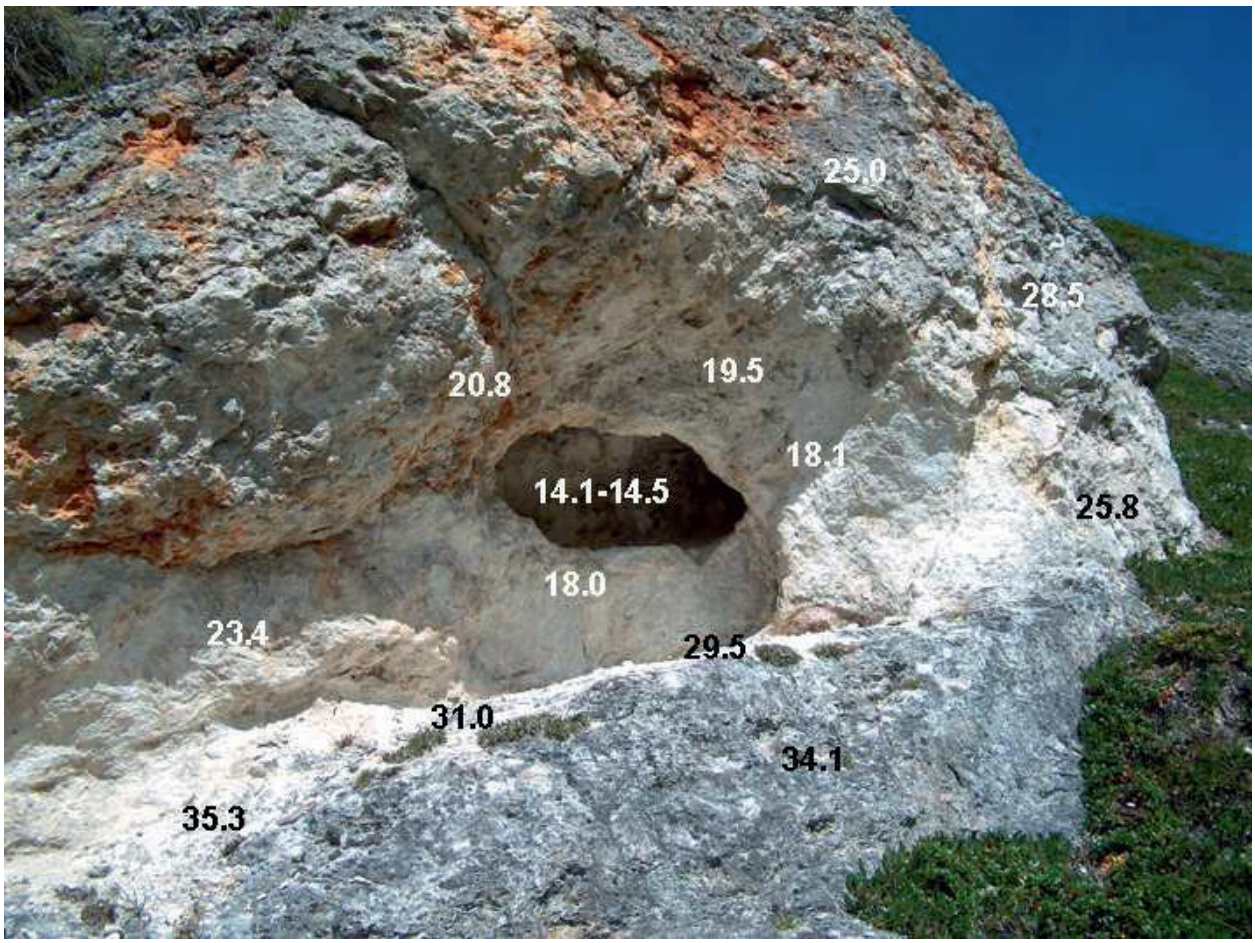

Fig. 6: Surface temperatures in degrees Celsius of the dolomite rock on 15.8.2004,15:15

1.5. Adaptations and strategies

One of the most effective adaptations of the organisms dwelling at extreme environmental conditions is to dry out. When desiccated, organisms even survive outer space conditions (Olsson-Francis et al., 2009). The production of a sheath composed of extracellular polymeric substances (EPS) around the bacterial cells belongs to this strategy. EPS first serve as a swamp and water reservoir and later with further desiccation as a tight protection crust around the cells. An important ability for the dried out organisms is to get back to normal life when the environmental conditions change to normal again. They very rapidly soak water and proliferate until the conditions get harsh again (Friedmann et al. 1974).

1.6. Small scale diversity of endolithic microorganisms

Investigations describing the microbial diversity in endolithic habitats are still ongoing. Recently Portillo et al. (2009) investigated three spots in short distance in a cave and found surprisingly different communities. Apparently, specific microorganisms grow and form a defined community due to the spatially different micro-environments. The

conclusion is that there are as many different microbial communities as there are different habitats!

1.6.1. Diversity detected by microscopy or by culturing

Table 2 lists some of the investigations conducted with classical methods. Jaag (1945) gives a comprehensive list. Golubic (1967) investigated endolithic organisms in the Dinaric Alps. His list is one of the most comprehensive ones.

One species seems to be universal, *Gloeocapsa*. This cyanobacterium may be the specialist for the endolithic habitat, or at least it is easy to detect. Other frequently found cyanobacterial genera are *Lyngbya, Calothrix, Schizothrix, Nostoc, Chroococcidiopsis, Chroococcus, Scytonema, Gloeothece, Synechocystis, Stigonema,* typical are furthermore the green algal genera *(Chlorophyta) Stichococcus, Trebouxia,* and *Chlorella.*

1.6.2. Diversity detected with molecular methods

Molecular techniques have increased rapidly the range of detected microbial species in any environment (Tab. 3). Amann et al. (1995) compare the culturability of bacteria from different environments such as seawater, freshwater, activated sludge or soil with the number of organisms found by molecular tools. The percentage of culturable bacteria of the total cell counts does not exceed at best 15% in activated sludge, but normally stays below 1% in most habitats. As extreme, it drops to 0.001% in seawater. Meanwhile new culturing methods have been developed to overcome this gap, but essentially these numbers still hold (Oren, 2004). Another surprising observation is that often the species detected by SSU rRNA gene sequencing are neither identical to those obtained by culture methods from the same environment, nor the most frequent observed by microscopy. Thus the two techniques, culturing and sequencing, deliver different and complementing results. In our study, none of the 96 sequences found was 100% identical with a sequence of a known cultured organism. The closest correspondence between a cultured organism and our sequences was for *Blindia acuta* with 99.7% similarity (Cox and Hedderson, 1999; accession number AF023681). Table 3 lists the accession numbers of the nucleotide sequences which are found at NCBI (http://www.ncbi.nlm.nih.gov/) searching for "endolithic", “cryptoendolithic” or “chasmoendolithic”, delivering a sum of 1875 entries deposited by 36 sources. Most of the investigations came from sandstone or limestone, including also the "sugar-grained dolomite" from the Piora Valley. Few matrices are granite, travertine, basalt, tuff, and gypsum. As long as the collected environmental samples are not utilized to cultivate the prokaryotes in parallel with the molecular analysis

of the SSU rRNA gene, one normally will not find sequences of previously cultivated organisms. Molecular surveys do not completely sample the genetic diversity of a community (Walker and Pace, 2007a and b). This actually shows the rich diversity in a single stone sample. It is even not sure whether we would find the same sequences again by restarting from the same sample. We certainly would find new clones. Another question is whether we would find more different species applying whole genome sequencing, or if we modify experimental factors that influence the result. Starting from the collection of the DNA, some cells get disrupted easily, others will resist like certain spores. Coextracted materials can cause problems during the DNA amplification; primers can be too unspecific or too specific, too short or too long leading to biased amplification or just different results. The annealing temperature may not be optimal for all primer sequences, the amplification time too short or the polymerase may decay too early. Only a quite intensive study may lead closer to the real picture of diversity in dolomite stones and other environmental samples.

1.6.3. Similarities and differences between the two lists

It is generally accepted (e.g. Garcia-Pichel and Castenholz 1991) that raw inocula from an untreated field sample placed into standard media usually only yields the fast-growing, opportunistic species that do not represent the important organisms in the original population. It is questionable for any environmental sample, whether one can really monitor by any presently available technique the whole community present. In soil only 1% of the bacteria present have been cultivated (Amann et al. 1995). Therefore it is not surprising that most of the prokaryotes newly detected by 16S rRNA sequencing are unknown, not yet cultivated, new species.

1.7. Atmospheric transport of microbes, astrobiology and biogeography

Biogeography is "to find and describe the distribution of biodiversity over space and time". Here on Earth the detection of endolithic microorganisms, the study of their distribution and abundance is proceeding with the goal to describe their biogeography. Do we find the same species composition at different places? Up to now one easily finds new species based on <97% similarity (Stackebrandt and Goebel, 1994; Stackebrandt and Ebers, 2006) of the SSU rRNA sequence at any new site. Sometimes sequences are fully identical to species that have been found elsewhere before, but more often sequences are slightly to massively (less than 95% sequence identity) different from what is saved in the

databank already. The present collection of available SSU rRNA gene sequences will continue to grow.

As microorganisms are transported on dust particles, dislocation within the whole world is possible. From the Sahara, dust clouds have been tracked from Africa to the North of Europe, bringing dust and bacterial spores to the snow fields in the Bernese Alps (Meier et al. 2005). Sahara dust also feeds the Carribean sea with nutrients. Darwin (1845, 1846) was among the first to find microorganisms in dust exported from the Sahara (Gorbushina et al., 2007). According to Griffin et al. (2002) between 10^{18} and 10^{20} microorganisms are transported annually through the atmosphere, making it difficult to imagine how topographic features of the Earth's surface could act as barriers for their dispersal. Other important transport vehicles for bacteria and archaea may also be humans, birds, and insects. Schlichting (1974) also counts fish to effective transporters of microorganisms but this does not apply for mountainous endolithic habitats.

A comparative 16S rRNA Analysis of bacterioplankton in the lakes Gossenkölle (Austria), Fuchskuhle (Germany), and Baikal (Russia) revealed globally distributed phylogenetic clusters including an abundant group of Actinobacteria (Glöckner et al., 2000). Another study about endospores of *Bacillus* species isolated from granite showed that *Bacillus* species are globally related considering the habitats of endolithic and extreme environments (Fajardo-Cavazos and Nicholson, 2006). As Baas Becking (1934) says: "Everything is everywhere – the environment selects", any given microbial species may occur and grow anywhere on the surface of the Earth provided that the particular habitat requirements are met. The distribution of microbes does not depend on contingencies occurring over evolutionary or ecological time scales as in the animal and plant world, but only on the properties of the habitat (Fenchel, 2005). Although some molecular data support the "cosmopolitan hypothesis" by showing a low genetic differentiation between populations separated by continental distances (e.g. Darling et al. 2000; Montresor et al. 2003), there is also contrasting data. Whenever there is a new habitat investigated one will find not only an "unsuspected high diversity" but also novel organisms with unknown close relatives (Logares 2006) as given e.g. by Lopez-Garcia et al. (2001), Moreira and Lopez-Garcia (2002), Moon-van der Staay et al. (2001), Dawson and Pace (2002) and Venter et al. (2004). On the basis of these results, it becomes apparent that the hypothesis proposing a general common global diversity for microbes does not agree with nature. We must assume that the real microbial diversity is much higher than recognized so far, and the next natural step would be to investigate patterns in the distribution of that diversity (Logares, 2006).

Norris and Castenholz (2006) state that the mechanism of the dispersal of endolithic microorganisms is unknown, but that exfoliation of rock surfaces that expose the microbial layer with subsequent dissemination by wind or insects may result in inoculation of other surfaces (e.g. Friedmann and Weed, 1987; Sun and Friedmann, 1999), but a gradual lateral spread within the rock may also occur (Van Thielen and Garbary, 1999). Indeed we observed that at our most used sampling spot in the Piora Valley the green layer always had reformed below the freshly cut stone latest by the end of a one year period. The colonization of neighboring space by microorganisms seems to work very efficiently.

Planets in our solar system might not be biologically isolated. The interplanetary transport of microbial passengers inside rocks, called "lithopanspermia" is presently being re-evaluated. Nicholson (2009) describes the probability for a microorganism to travel from one planet to another as a product of several probabilities which consist of:

1. The impacting object strikes a biologically inhabited zone.
2. The ejection of rocks with endolithic microbes onto an escape trajectory.
3. An organism survives the launch.
4. Survival of space transit.
5. Surviving entry through the recipient planet's atmosphere.
6. Surviving impact onto the recipient planet's surface.
7. Release from the interior of the rock.
8. Survival and proliferation in the new environment of the recipient planet.

Given all these probabilities are above zero, it is theoretically possible for a microorganism to sustain an interplanetary trip. Different groups analyzed this probability and came to opposite conclusions – that interplanetary transport of microbes was either highly probable (Mileikowsky et al., 2000) or highly improbable (Clark, 2001). Already to think about the possibility that life could be transported from one planet to another is outraged, but how can we calculate it? One important argument against lithopanspermia is that the energy required to eject rocks from the surface of a planet into space would be so high that it will partially melt or vaporize the rock, thus rendering it sterile (Mileikowsky et al., 2000; Clark, 2001). In contrast, it has been recognized that several meteorites found on Earth are actually fragments of crust derived from the Moon and Mars (McSween, 1994). Because of heat-labile carbonates and magnetic signatures found in them, it is estimated that many of

the Martian meteorites suffered only light shock pressures and some were not heated above about 100℃ when being boosted into space (We iss et al. 2000; Shuster and Weiss, 2005).

For the transport of samples of Martian crust to Earth physicists propose a model which describes a "spallation mechanism for impact ejection" where rocks can be launched into space with relatively little damage (Melosh, 1984; 1985; 1989; Gratz, et al. 1993). By this way, a transient spallation zone forms around an impact site, where the reflected shock wave of the impact is directly translated into acceleration of surface rocks to escape velocity. Because the spallation zone penetrates at most only a few meters into the surface and most of the Martian meteorites are igneous (mainly basalt), it becomes relevant to understand the microbial ecology of near-surface igneous rocks on Earth (basalts and granites) which might harbor potential microbial candidates for interplanetary transfer (Nicholson, 2009). After all, dolomite does not belong to the category of hard stones and therefore it may be excluded from potential vehicles for interplanetary transports. Nevertheless, if the material gets surrounded with a shielding shell, it may still serve as a vesicle.

Comets may also serve as vehicles of life. Recently NASA scientists discovered the amino acid glycine in samples of comet Wild 2 (dense gas and dust surrounding the ice nucleus of Wild 2) returned by NASA's spacecraft Stardust on January 2, 2004 (http://stardust.jpl.nasa.gov/news/news115.html). Recently also the survival of spores and dried bacteria in space was tested in a space flight in Low Earth Orbit (LEO) for 10 days (Olsson-Francis et al., 2009).

1.8. The Dolomite Problem

The exact mechanism of the formation of dolomite [calcium magnesium carbonate, $CaMg(CO_3)_2$] in the geological past and present remains despite much research a subject of debate and is often referred to as "The Dolomite Problem" (McKenzie, 1991). Vast deposits of dolomite are present in the rock record, with fluctuating but globally decreasing abundance throughout the geological time. This uneven distribution of dolomite suggests the existence of a link between global environmental changes and dolomite formation (Bontognali, 2008). However, this link and its significance have not been established yet. In addition laboratory synthesis of dolomite under sterile conditions has only been possible at temperatures greater than 100℃ (Land, 1998; Lip pman, 1973). These temperatures are typical of burial in sedimentary basins, even though much dolomite in the rock record

appears to have been formed under low-temperature conditions (McKenzie, 1991). Bontognali (2008) now proposes a new model of dolomite formation:

As dolomite occurs in association with surface and buried microbial mats, dolomite may precipitate as a consequence of mineral nucleation within the extracellular polymeric substances (EPS) constituting the mats. EPS molecules seem to act as an organic template, and influence the elemental composition of the precipitate leading to dolomite formation. This process is observed in surface microbial mats, but it is even more pronounced in 1000 years old microbial mats, which are buried in the supratidal zone and no longer show signs of metabolic activity.

This led to the hypothesis that EPS, rather than an active microbial metabolism, is the major factor responsible for the mineral forming process. This interpretation differs from current models, in which microbial dolomite formation is mainly linked with an increase in pH and the consumption of sulfate due to metabolic activity. The key role of EPS in dolomite formation was tested in laboratory culture experiments. Mg-calcite and Ca-dolomite were successfully precipitated at 30℃ in the presence of freshly produced EPS, while the bacteria present had been inactivated with antibiotics (Bontognali, 2008).

1.9. ARB – a tool to monitor phylogeny

More than two decades ago the taxonomy of bacteria and archaea became strongly supported by the sequence information of the small and the large subunit (SSU and LSU) of the ribosomal RNA gene. Starting with the exploring studies of Pace, Olsen, Giovannoni and Ward (Pace et al. 1985; Olsen et al. 1986; Giovannoni et al. 1988; Ward et al. 1990) the ribosomal RNA molecule has been established as the "gold standard" for the investigation of the phylogeny and ecology of microorganisms (Woese, 1987; Amann et al., 1995; Pace, 1997; Stackebrandt and Ebers, 2006). Today, over one million publicly available SSU and LSU rRNA gene sequences have to be put in their right position in the phylogenetic tree by means of appropriate software tools and special quality controlled databases. The ARB program is one of the programs developed for this purpose about 15 years ago and is under continuous improvement and further development according to the demand to handle the steadily growing amount of data (Ludwig et al. 2004). ARB offers a graphical user interface and a broad palette of interacting programs built around an independent database. Although several other databases exist, the ARB project is currently the only one which incorporates homologous SSU rRNA gene sequences of Eukaryotes (18S rRNA gene) and offers LSU rRNA gene databases (23S and 28S rRNA genes) including sequences from all three domains of life. ARB was originally developed

for UNIX systems and now also runs on LINUX or Mac OSX. With the program package a downloadable database can be opened and enriched with new DNA sequences. New databases can also be created with amino acid sequences. Starting with a minimum of three aligned sequences, new sequences are easily imported and automatically aligned. Since it is necessary to build phylogenetic trees on a proper sequence alignment, emphasis should be put on an accurate alignment, manually controlled with a critical eye (Ludwig and Schleifer, 1994). One of the advantages of ARB is that all these steps are made in its environment without using additional programs. Another advantage is the fast automatic alignment, which is achieved by the invention of a so called PT Server. This Server holds the entire sequence database in form of 20-mers but duplicated starting from every base. These 20-mers allow a much faster comparison between the different sequences and increase the speed of the calculation. For the final tree construction three methods are included in the ARB software package: Distance Matrix (Neighbor Joining), Maximum Parsimony, and Maximum Likelihood. Further, ARB not only serves as a tree former but also to find and design specific oligonucleotide probes.

To clearly separate two different species, normally the DNA-DNA-Hybridization procedure (DDH) is used and a value higher than 70% reassociation has been defined as the threshold value indicating that the two compared genomes belong to the same species (Wayne et al., 1987; Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001). With the growing availability of complete genomes new approaches for phylogenetic analyses have been found. The Multi Locus Sequence Typing (MLST) is a method for the genotypic characterization of prokaryotes at the infra species level, using the allelic mismatches of a small number (usually 7) of housekeeping genes (Gevers et al., 2005). Similarly the Multi Locus Sequence Analysis (MLSA) compares the sequences of multiple protein-coding genes, normally also artificially concatenated, and is used on the inter species level (Gevers et al., 2005). The quality (measured in bootstrap values) of a phylogenetic tree generated with MLST or MLSA varies depending on the number of genes used: Below eight gene sequences can result in unstable topologies, above twelve are necessary to reach the threshold level of reliability (Soria-Carrasco et al. 2007). Interestingly, when using large concatenates, Multi Locus Sequence Analysis gives identical topologies as the SSU rRNA gene sequence tree reconstructions (Soria-Carrasco et al. 2007). With Multi Locus Sequence Typing, the advantage of getting a higher resolution of the phylogeny so far costs more time and money compared to the SSU rRNA approach. A third technique is the Average Nucleotide Identity ("ANI", Konstantinidis and Tiedje, 2005a; Goris et al.,

2007). Values of about 94% ANI correspond to the traditional 70% DNA-DNA reassociation standard of the current species definition (Konstantinidis and Tiedje, 2005a). Recently, Richter and Rossello-Mora (2009) using the software package JSpecies found it to be a user-friendly, biologist-oriented interface to calculate the Average Nucleotide Identity. They narrowed the boundary of ANI from 94% to ≈95-96% to substitute DNA-DNA-Hybridization and suggested that for taxonomic purposes a random sequencing of at least 20% of the genome of the strain in question is sufficient to assign the correct species name. Yet, the fact, that the traditional 16S/18S rRNA gene sequence will deliver about the same result as e.g. MLSA with 22 housekeeping genes is calming, especially in a time and in countries where money plays an increasingly important role.

Published in: *Microbial Ecology* (2006) **51**:353-364

(2.) An Endolithic Microbial Community in Dolomite Rock in Central Switzerland: Characterization by Reflection Spectroscopy, Pigment Analyses, Scanning Electron Microscopy, and Laser Scanning Microscopy

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Keywords

endolithic phototrophic organisms, cyanobacteria, dolomite, spectroscopy, bacteriochlorophyll, light penetration, lectin, confocal laser scanning microscopy, two-photon laser scanning microscopy, EPS

Abbreviations

PAR - photosynthetic active radiation, UV - ultra violet, EPS – extra cellular polymeric substances

Abstract

A community of endolithic microorganisms dominated by phototrophs was found as a distinct band a few millimeters below the surface of bare exposed dolomite rocks in the Piora Valley in the Alps. Using *in situ* reflectance spectroscopy, we detected chlorophyll a (Chl *a*), phycobilins, carotenoids, and an unknown type of bacteriochlorophyll-like pigment absorbing *in vivo* at about 715 to 720 nm. In cross sections, the data indicated a defined distribution of different groups of organisms perpendicular to the rock surface. High-pressure liquid chromatography analyses of pigments extracted with organic solvents confirmed the presence of two types of bacteriochlorophylls besides chlorophylls and various carotenoids. Spherical organisms of varying sizes and small filaments were observed *in situ* with scanning electron microscopy and confocal laser scanning microscopy (one- and two-photon technique). The latter allowed visualization of the distribution of phototrophic microorganisms by the autofluorescence of their pigments within the rock. Coccoid cyanobacteria of various sizes predominated over filamentous ones. Application of fluorescence-labeled lectins demonstrated that most cyanobacteria were embedded in an exopolymeric matrix. Nucleic acid stains revealed a wide distribution of small heterotrophs. Some biological structures emitting a green autofluorescence remain to be identified.

Introduction

Endolithic phototrophic communities occur worldwide and are particularly important as pioneers in cold and hot dry habitats. In the Piora Valley, such organisms colonize bare dolomite rock and form a clearly defined layer a few millimeters below the rock surface. Studies of endolithic microorganisms started with Diels [7], who discovered this phenomenon in the Dolomites in Austria and Italy. Since then endoliths have been described in habitats like hot semiarid lands, rocks in streams, and hot and cold deserts (for recent reviews, see [1, 10, 16, 34, 38]). Endolithic populations are dominated by cyanobacteria and surprisingly, the endolithic cyanobac- terial biomass is estimated to amount to 5-6% of the total global cyanobacterial biomass [15]. So far, most of our knowledge of endolithic microorganisms is restricted to oxygenic phototrophs (green algae and cyanobacteria) and fungi as partners of lichen symbiosis. Recently, endolithic populations have gained new interest because they may serve as a model of extraterrestrial life, e.g., life on Mars [20, 22].

Characterization and taxonomic determinations of endolithic organisms were done mainly by light and electron microscopy, and culturing. Only recently have new

spectroscopic methods been introduced [29, 37], and molecular methods have been used rarely up to now [5, 30]. In most investigations, the spatial arrangement of the endolithic population was not resolved, as the samples prepared from the rock average both horizontally and vertically.

The objectives of this work were (1) to determine the spatial heterogeneity of the phototrophic endolithic population and (2) to obtain information on its structure perpendicular to the rock surface by investigating the intact endolithic population in dolomite rock by analyzing the distribution of pigments and cells with *in situ* techniques, reflection spectroscopy, and confocal laser scanning microscopy [CLSM, and two-photon (2P)-LSM].

Methods

Source of the Material. Dolomite rock was collected in the Piora Valley in the southern part of the Swiss Alps at an elevation of 1965 m a.s.l. The coordinates of the specific sampling site are 46°33' N, 8°43' E (Swiss coordinates, 698 100/155 950). The geology of the Piora Valley is characterized by a dolomite trough, oriented east-west and embedded with crystalline rock formations. Due to erosion, the dolomite is often not covered by vegetation and exposed to the atmosphere, forming white cliffs. Mean annual temperature in the Piora region is between 0°C and 5°C, with a precipitation of approximately 150 cm per year and an average sunlight intensity of 150 W m^{-2} [30]. The sampling site is characterized by the presence of a horizontal hole of about 60 cm x 80 cm with a depth of 60 cm. The rock surface inside the cavity is protected from direct light and rain, whereas the outside is exposed to full sunlight and weather conditions. This allowed us to obtain samples from different light regimes. Rock pieces of some millimeters to centimeters in size were split off the surface with chisel and hammer and kept in sterile Falcon tubes or petri dishes at 4°C in the dark until prepared for the various experiments in the laboratory.

Field Methods. Surface temperatures of rocks were obtained with an infrared reflection thermometer.

Measurement of Light Attenuation. Rock pieces of about 5 cm x 5 cm in size were cut to flat plates of about 5-mm thickness and bore holes of 8-mm diameter, and various depths were drilled into them by using a Dremel Moto tool (Dremel, Racine, WI, USA) as described by Matthes *et al.* [21]. The thickness of the remaining plate was

measured with a caliper (accuracy, 0.05 mm). Transmitted light was measured with a PDA1 photodetector amplifier and the VIS Fiber Optic Detector (World Precision Instruments, Sarasota, FL, USA). Illumination of the rock surface by visible light was achieved either by diffuse neon light or by a beam perpendicular to the surface from a light fiber coupled to a tungsten 12-V light source.

In Vivo Spectroscopy. Rock pieces were carefully cut rectangular to the surface, flattened with a grinding stone (Dremel Moto tool), and fixed on a mechanical positioning table such that the endolithic band was parallel to its Y axis and the flattened side of the rock to be scanned was parallel to the table plane. The tip of the fiber optic was coupled to the Labspec VNIR-512 diode array spectrometer (Analytical Spectral Devices, Boulder, CO, USA) and two fibers for the illumination of the rock were mounted vertically above the rock surface resulting in a light spot of about 2 mm in diameter. Details of the system have been described by Wiggli *et al.* [35]. A series of reflection spectra were collected in steps of 0.5 mm in a right angle across the endolithic band with an acquisition time of 8.7 s and a spectral resolution of 3 nm with a sampling interval of 1.4 nm. Data were imported into Excel. As the noise level increased below 450 nm and above 750 nm, only the data in between these values were used for calculations. The spectra were normalized at 750 nm. A silica thin-layer chromatography plate served as white reference and a reflection spectrum obtained from a freshly cut site from a location deep in the rock was used for baseline correction. The pigment concentration was calculated for chlorophyll a (Chl *a*) by taking the absorption at 680 nm directly. For bacteriochlorophyll-720 (Bchl-720) the absorption at 720 nm was corrected empirically for the absorption of Chl *a* in the range between 700 and 750 nm. The phycobilin concentration was calculated from the absorption at 620 nm, corrected for the absorption of Chl *a*.

Absorption Spectroscopy (with MeOH) and Pigment Analyses (with Aceton) by High-Performance Liquid Chromatography. 30 g of Dolomite rock scratched from the microbial zone was ground in a mortar and extracted with methanol or 5 ml acetone (100%). The extract was decanted and its spectrum was measured on a Uvicon 860 spectrophotometer (Kontron, Zürich, Switzerland). After sedimentation of the sandy material, the supernatant was concentrated under a nitrogen gas flow. The solution was passed through a 0.2-μm particle filter and 500 μL was used for high- performance liquid chromatography (HPLC) analysis on a Shimadzu 10AVP System equipped with a C-18 Grom-Sil 120 ODS-4HE reversed-phase column (4.6 mm x 250 mm, 5-μm particle size,

Stagroma, Germany) and a photo diode array detector. The flow rate was 1 mL min^{-1} at 30℃. The solvent gradient ranged from 0% to 100% s olvent B during the first 10 min, then kept at 100% solvent B for additional 30 min [solvent A: methanol/H_2O 80:20 (v/v), solvent B: methanol/acetone 80:20 (v/v)]. For identification standards, Chl *a* and Chl *b* were purified from *Scenedesmus subspicatus*, and bacteriochlorophylls from *Chlorobium limicola* and *Allochromatium warmingii*.

Microscopic Methods

Light Microscopy. A Wild M7 binocular (Wild, Heerbrugg, Switzerland) was used for the measurements of the width and location of the bands; photographs were taken with a Nikon Coolpix 990 digital camera and a microscope adapter (Nikon MXA 29005).

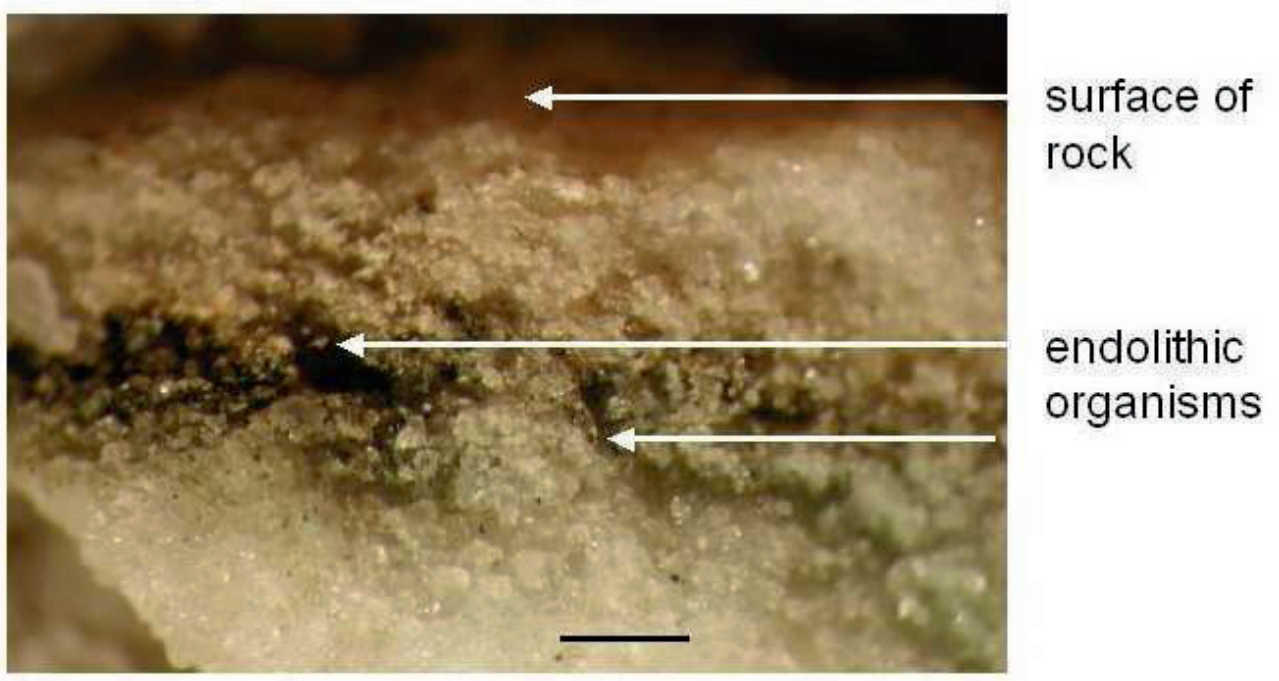

Figure 1. Macroscopic image of the endolithic layer of organisms in broken dolomite. Bar=1 mm.

Scanning Electron Microscopy. For scanning electron microscopy (SEM), rock pieces as prepared for reflection spectroscopy were sputter-coated with gold and examined with a Sn-4100 FSEM scanning electron microscope (Hitachi, Tokyo, Japan) at 20 kV.

Laser Scanning Microscopy. Confocal laser scanning microscopy (CLSM) and two-photon laser scanning microscopy (2P-LSM) was done with a Leica TCS SP MP attached to an upright microscope (Leica, Heidelberg, Germany). The system was equipped with a set of lasers: argon (458, 476, 488, 514 nm), krypton (568 nm), helium-

neon (633 nm), and titanium/sapphire infrared (760-900 nm). The microscope was controlled by the Leica Confocal Software Version 2.00 Build 0871. Images were collected with 20 x 0.5 NA, 63 x 0.9 NA and 63 x 1.2 NA water-immersible lenses (Leica) in the Z direction for subsequent image analyses. Images were presented as multichannel, maximum-intensity projections using the microscope software. In addition, 3-D isosurface projections were calculated by using Imaris in combination with the Surpass tool (Bitplane, Zürich, Switzerland). Rock pieces were prepared similarly as for reflection spectroscopy. Pieces of about 5 mm x 5 mm in size were mounted on microscopy slides and studied while submersed in water. Nonspecific nucleic acid staining was carried out using DAPI (Fluka, Buchs, Switzerland), Syto 9, and Syto 40 (Molecular Probes Inc., Eugene, OR, USA). Glycoconjugates in the extracellular polymeric substances (EPS) matrix were stained with Alexa-488 (Molecular Probes) fluorescently labeled *Aleuria aurantia* lectin (Vector, Burlingame, CA, USA) as described previously [23].

Results

Description of Rock Material and Endolithic Organismic Bands. In exposed dolomite, endolithic microorganisms are easily observed when the rock surface is mechanically removed. These organisms form a gray to green layer a few millimeters below the rock surface (Fig. 1). The depth of the band and its thickness are variable and probably determined by the average light intensity at the specific site. From 80 samples collected randomly both from sites exposed to sunlight and from shaded ones, depth and thickness of the band were measured. Both values exhibited broad variations. However, the distance of the endolithic organisms from the surface was significantly larger ($P<0.0001$) at sunlight-exposed sites than at shaded ones: 2.27 ± 0.82 mm and 1.01 ± 0.51 mm, respectively. Similarly, the width of the band was wider under high light compared to low light: 1.77 ± 0.52 mm and 1.08 ± 0.37 mm, respectively. Extremes for the distance from the surface for high-light sites were 0.5 mm and 4.8 mm; for low light sites, 0.3 mm and 2.0 mm. Besides the differences in the light regime, the temperature of rock surfaces exposed to sunlight was regularly 5℃ to 15℃ above the ambient air temperature, whereas the daily fluctuations at the constantly shadowed sites were less than 5℃.

Light Attenuation by Dolomite Rock. Figure 2 summarizes the light attenuation in visually homogeneous dolomite rock. The data points fit to an exponential curve, which, however, does not extrapolate to 100%. Light entering perpendicular to the rock surface penetrated deeper into the rock as compared to diffuse light where less than 5% of the surface intensity was measured at 1 mm within the rock.

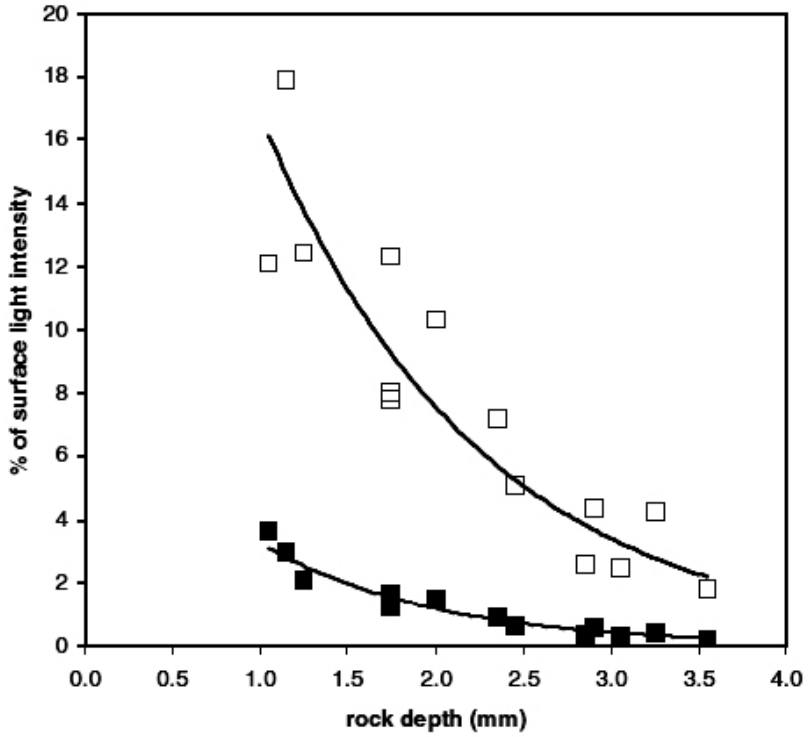

Figure 2. Light attenuation by dolomite rock in percent of surface light intensity. *Open squares*, illumination with perpendicularly oriented light from fiber optic 1 cm away from the rock surface. *Closed squares*, illumination with diffuse light from 2-m distant fluorescent tubes.

Reflection Spectroscopy. Figure 3 shows two selected *in vivo* spectra from different samples, taken at the maximum pigment concentration within the band. Both spectra are characterized by the typical absorption regions of cyanobacterial pigments, Chl *a* at 680 nm and phycobilins at around 620 nm. In addition, spectrum (b) has a distinct shoulder at about 720 nm (or 715 nm?), suggesting the presence of a bacteriochlorophyll typical for green phototrophs.

The pigment distribution across the endolithic band was determined from a set of spectra taken at intervals of 0.5 mm from the surface, using the calculations for Chl *a*, phycobilins and Bchl-720 as described in "Methods". When repeated at the same site, the reflection spectra were fully reproducible. In contrast, scanning at other places of the same band or moving along the direction of the band parallel to the surface resulted in a substantial pigment microheterogeneity, which was not expected at first. Especially, the

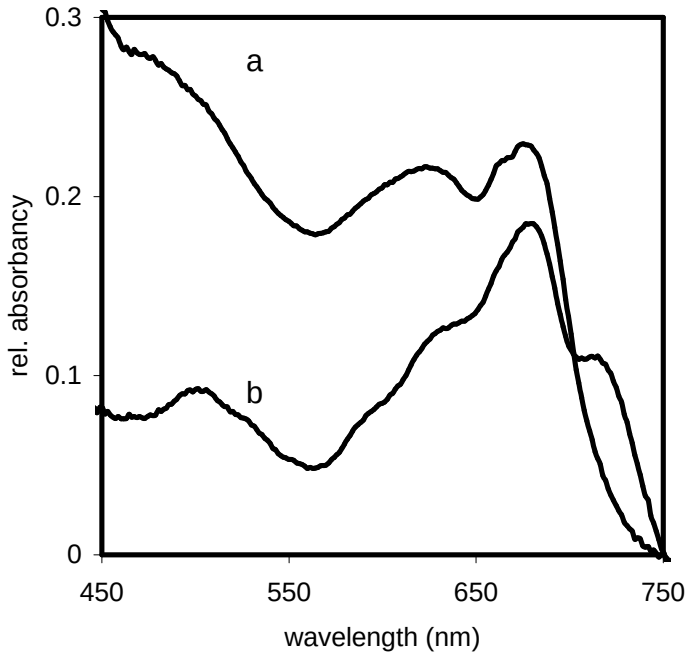

Figure 3. Examples of *in vivo* reflection spectra from the center of endolithic bands. (a) Site with high phycobilin content but no Bchl 720. (b) Site with low phycobilin content but with Bchl 720.

size of the 720-nm absorbing shoulder and the relative amount of phycobilins were highly variable. Figure 4 presents the distribution of the pigments across the endolithic layer for two different sites. Most pigment transects showed two peaks in Chl *a* concentration across the band. High-light samples normally lacked the absorption at 720 nm (Fig. 4a). Inside the rock, the phycobilin to chlorophyll ratio increased with depth (Fig. 4a). When present, the Bchl-720 band was situated deep in the rock coinciding with the second peak of Chl *a*; phycobilins were scarce at these sites (Fig. 4b). Often, a peak at 660 nm was present at the outer side of the endolithic band, suggesting the presence of free chlorophyll in denatured cells (see Fig. 3a).

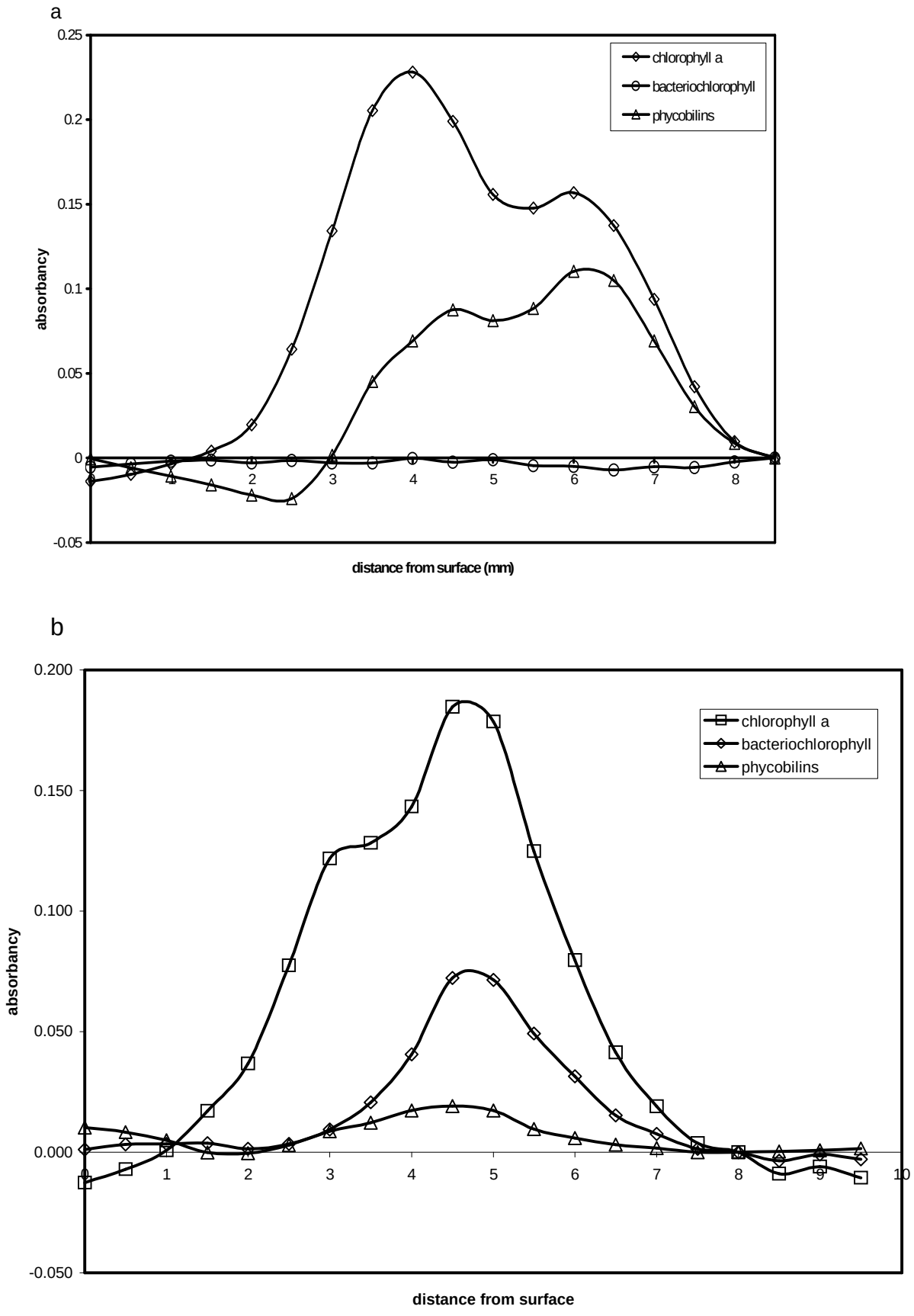

Figure 4. Pigment profiles through endolithic bands given as absorption values calculated from the reflection spectra. (a) Site with high phycobilin content but no Bchl 720. (b) Site with low phycobilin content but with Bchl 720.

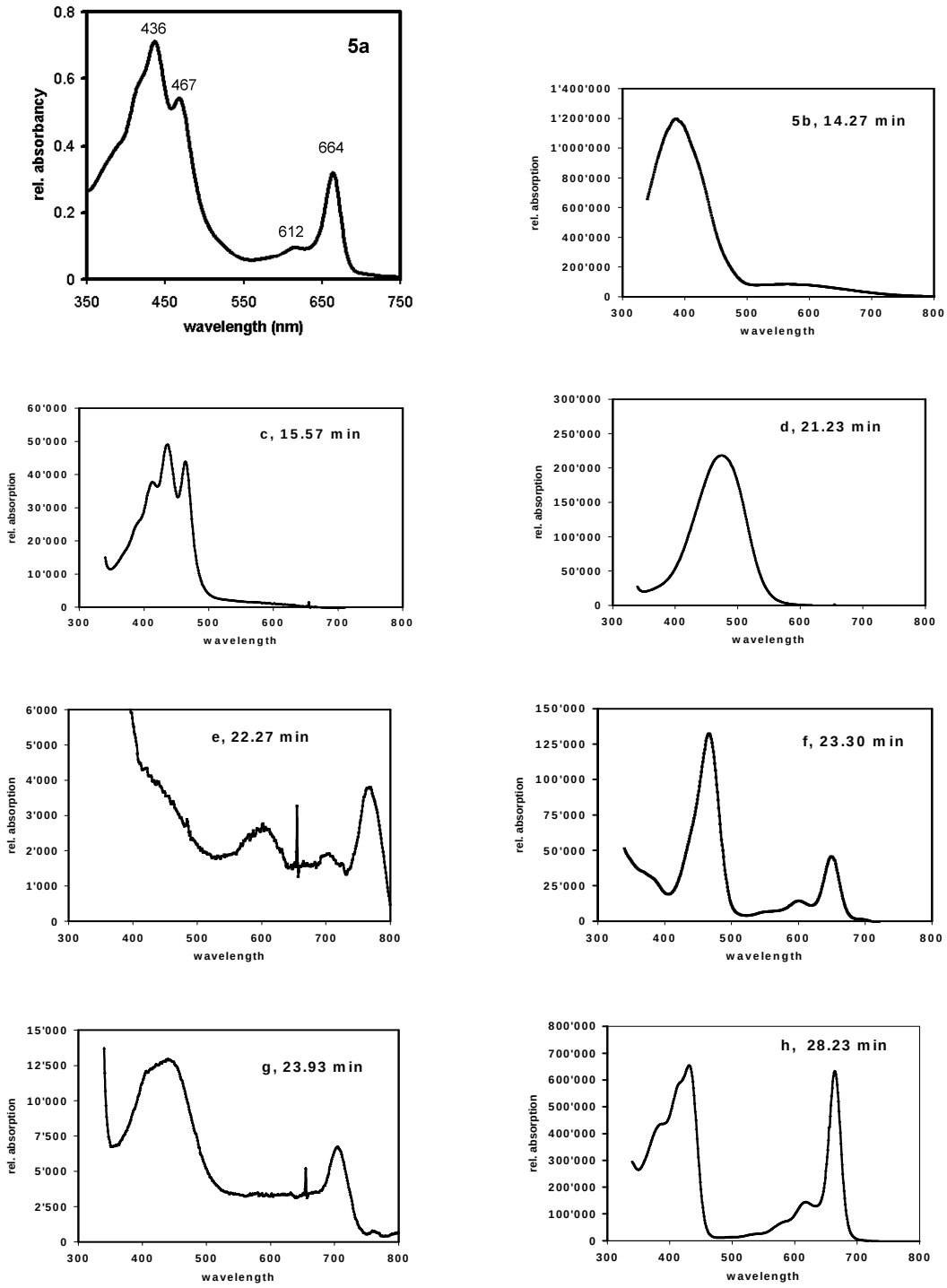

Figure 5. Absorption spectra of the raw methanolic extract of endolithic rock material (a) and of selected compounds after HPLC separation (b-h). Numbers given indicate the time of appearance during chromatography.

Pigment Analyses. The absorption spectrum of an extract of the endolithic band in methanol shows the properties of the dominating Chl *a* with maxima at around 436 nm and 664 nm. No absorption was observed above 700 nm; thus, organisms containing Bchl *a* or

Bchl *b* may only be present in minor quantities. A third maximum at around 467 nm possibly originated from other pigmented organisms in the bacterial layer (Fig. 5a). Separation of the lipophilic compounds by HPLC yielded a sum of absorption spectra, some of which were identified. A compound with λ_{max} of 387 nm eluted early (Fig. 5b); it showed a high absorption, and its spectrum suggested a scytonemin-like UV-shielding pigment [12].

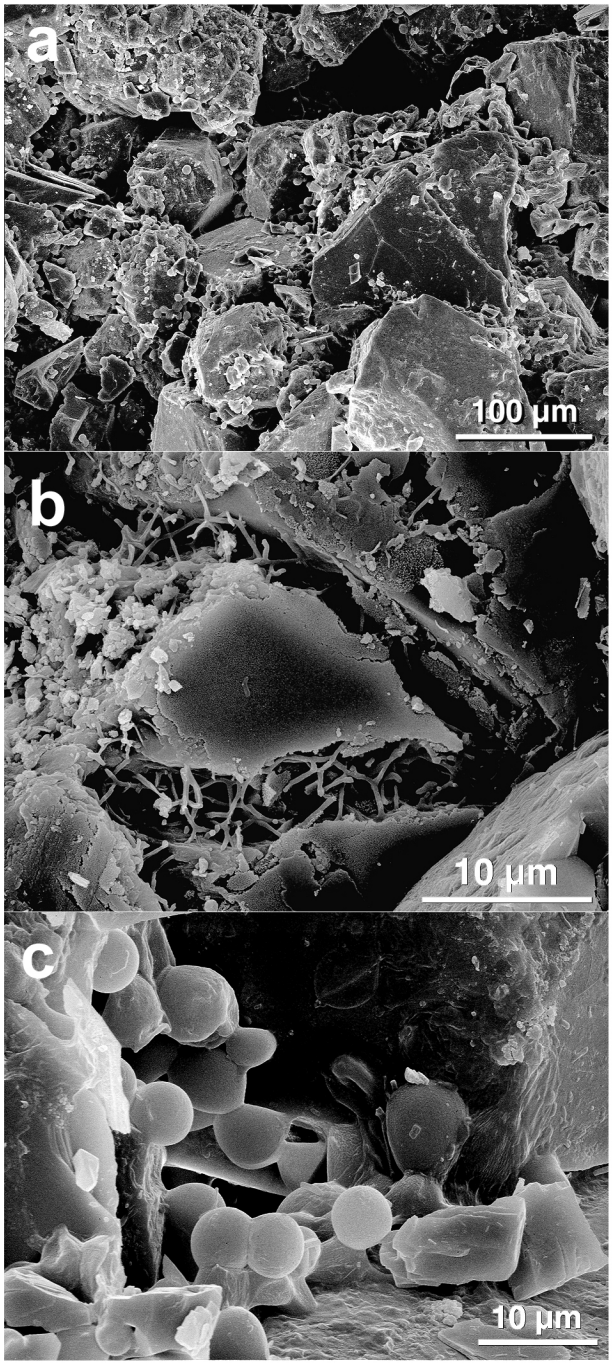

Figure 6. SEM images from endolithic organismic zone. (a) Low- magnification overview showing single coccoid cells. (b) High magnification showing filamentous cells. (c) High magnification showing budding-like structures.

Several carotenoids with the typical three absorption maxima followed (example in Fig. 5c), as well as an unknown compound with a broad band between 450 and 500 nm without specific structures (Fig. 5d). A compound with maxima at 466 nm and 650 nm (Fig. 5f), indicating Chl *b*, appeared after 23.3 min. Finally, Chl *a* eluted after 28 min in high concentration (Fig. 5h). Two spectra were obtained with chlorophyll-like absorption properties, both with defined absorption bands at or above 700 nm (Figs. 5e, g), the former indicative for Bchl *a*.

Microscopy. SEM images allow one to describe morphotypes and to estimate roughly the dimensions of the structures, but not to get information on the spatial organization of the organisms within the band. Three selected images illustrate bacterial colonization in the dolomite. Figure 6a shows a wide distribution of single coccoid cells of about 5 µm in diameter attached to surfaces, Small globules (<0.5 µm) forming aggregates cover grains of rock, and fine

filamentous structures of 0.3 μm to 0.5 μm in width, lead to a network in the crevices of the rock (Fig. 6b). In Fig. 6c, large spherical cells of about 5 μm to 7 μm in diameter are observed that seem to grow by budding. Traces of interactions between living organisms and inorganic surfaces, such as biocorrosion or calcite formation, were not observed.

CLSM (one-photon excitation) was used for imaging in the reflection and fluorescence mode. The reflection mode allowed recording of reflective signals originating from inorganic solid material, e.g., rock compounds or precipitates (see Figs. 7, 8, 9). The fluorescence mode was used directly without staining to record the autofluo- rescence of the organisms. For this purpose, the samples were excited with three laser lines (488, 568, and 633 nm) and the resulting emission signals were recorded in the green (500-550 nm), red (575-625 nm) and far red (650-800 nm) channel. Thereby, the autofluorescence of phycobilins is detected in the red channel, whereas the Chl *a* signal is recorded in the far-red channel. The fluorescence mode was also used after staining with nucleic-acid-specific fluorochromes and glycoconjugate-specific lectins labeled with Alexa-488. LSM with two-photon excitation was used to confirm the results from one-photon excitation and to take advantage of imaging deep regions of the samples.

At low magnification, five CLSM images were taken perpendicular to the surface to form a cross section, showing again the spatial heterogeneity of the phototrophs (Fig. 7). The autofluorescence was quantified to demonstrate the distribution of both Chl *a* and the colocalized signal of cyanobacterial pigments. The result showed a high Chl *a* signal in the outer region of the rock, whereas the cyanobacterial signal in this sample was highest at about 6 mm depth. Both signals decreased with depth corresponding to the measurement of light intensity within dolomite rock (Fig. 7).

Coccoid cyanobacteria were present not only as single cells, but also as multicellular aggregates. The size of single cells varied between 3 μm to 6 μm in diameter. At other locations, spherical, baglike structures of 15 μm to 30 μm in size were found (Fig. 8). Some of them clearly contained cyanobacterial cells identified by the colocalized autofluorescence (Figs. 8a, b). Other spheres contained particles of cell size but emitting a green autofluorescence (Figs. 8a, c). The sheaths around the spheres with cyanobacterial cells and green particles emitted a green autofluorescence as well. This result was obtained after one-photon excitation at 488 nm, emission channel set to 500-550 nm (Figs. 8a-c), as well as by two-photon excitation at 800 nm, emission channel set to 400-575 nm (Figs. 8d, e).

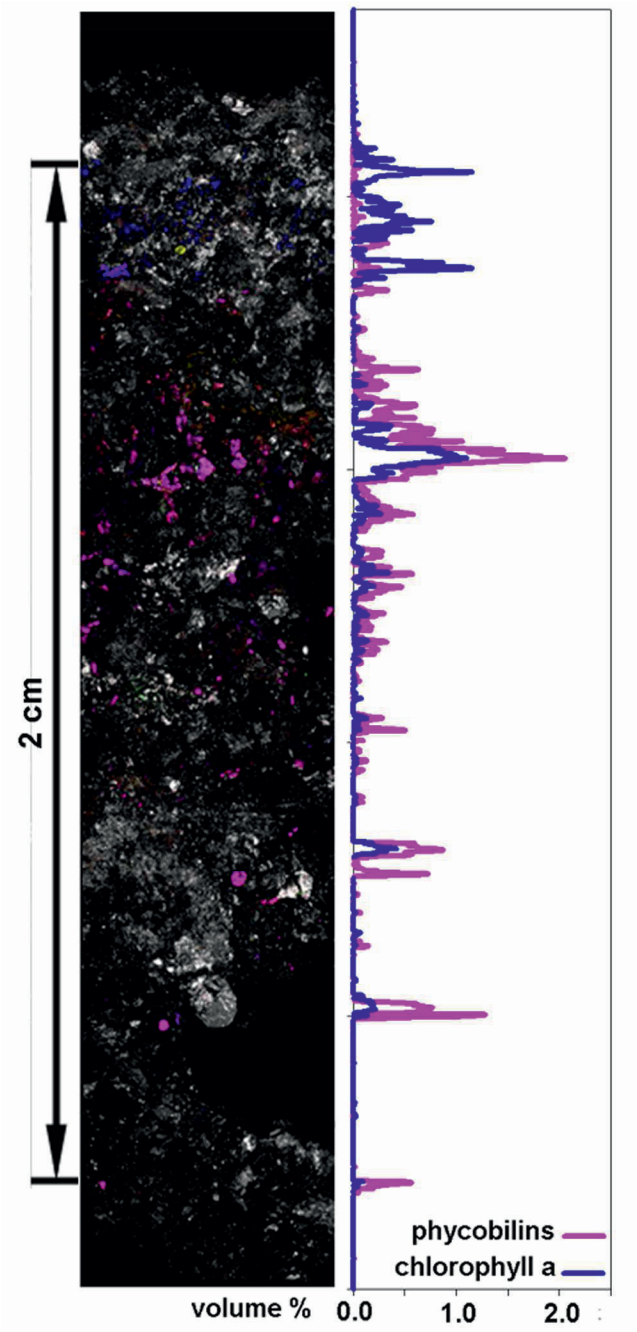

Figure 7. CSLM profile through outer part of dolomite rock with endolithic zone of phototrophic microorganisms and quantification of the autofluorescence signals. The image is composed of five single image series. Volumes were calculated from each of the five image stacks corresponding to the various depths. Color allocation: reflection, white; Chl *a*, blue; phycobilins, pink.

At certain locations, bundles of flexible filaments were observed having an autofluorescence signal similar to the coccoid cyanobacteria (Fig. 9a). Even at higher magnification the filaments did not show any structural details and no further taxonomical determination was possible.

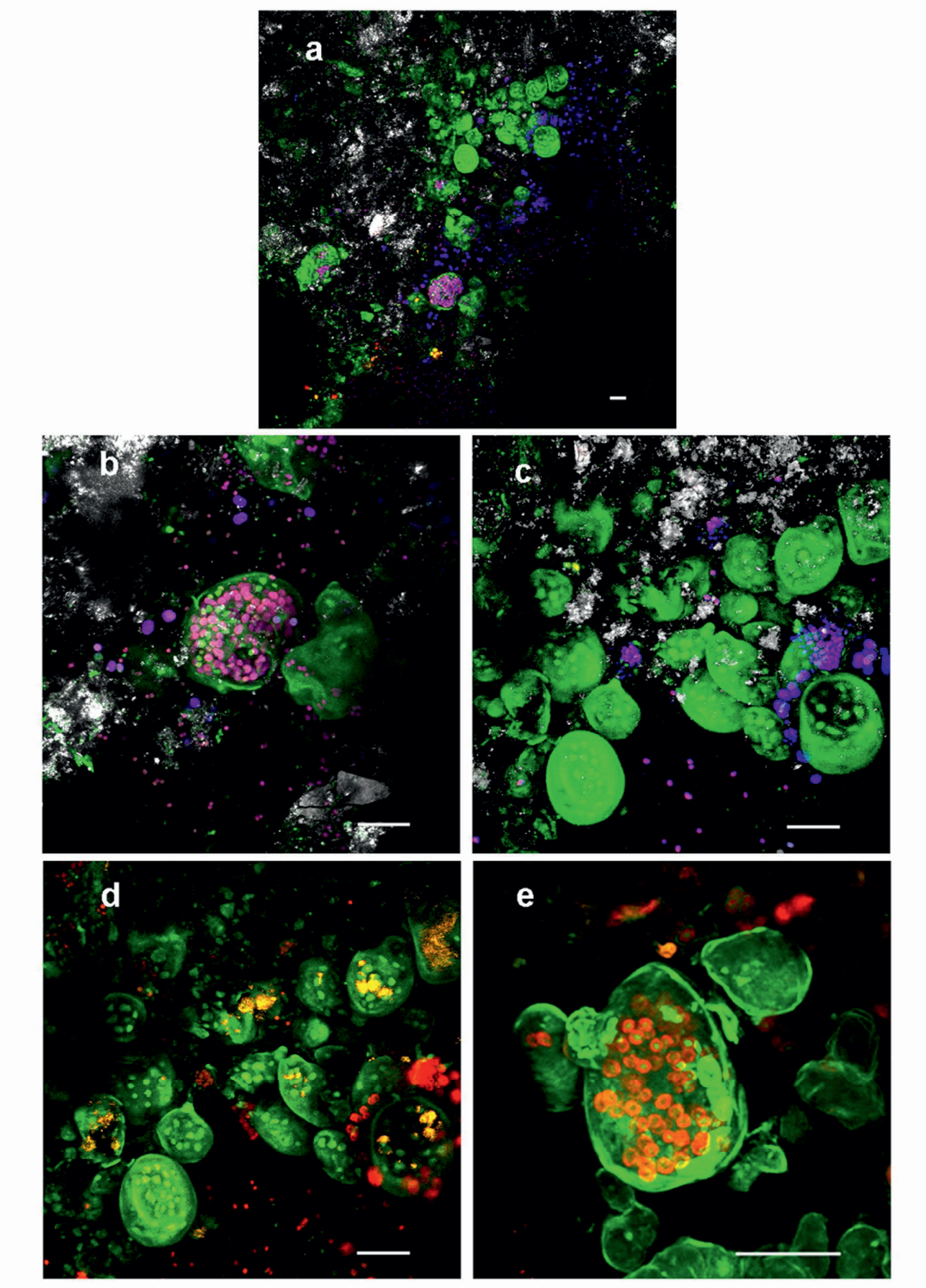

Figure 8. Maximum intensity projection of LSM images from endolithic microbial community showing locations with baglike structures (bars=20 mm). (a) One-photon LSM after excitation with three visible lasers and record of signals in four channels. Color allocation: reflection from minerals, white; unknown green

autofluorescence, green; colocalized autofluorescence of cyanobacteria in the red and far-red channel, pink (red/blue); autofluorescence of Chl *a* in the far-red channel, blue. (b) Detail of image (a) at higher magnification with pink microcolony surrounded by a distinct envelope showing green autofluorescence. Color allocation as in (a). (c) Detail of image (a) at higher magnification with single blue coccoid cells and green micro- colonies surrounded by a distinct envelope showing also a green autofluorescence. Color allocation as in (a). (d) Two-photon LSM of baglike structures with excitation at 800 nm and dual-channel recording of autofluorescence (green emission, 400-575 nm; red emission, 600-750 nm). (e) Two-photon LSM of baglike structures with excitation at 800 nm and dual-channel recording of autofluorescence and DAPI staining (green emission, 400-550 nm; red emission, 600-750 nm). DAPI stained the cell interior of the cyanobacteria within the baglike structure only, whereas the other green signal corresponds to the green autofluorescence of the envelope material.

Experiments with the life nucleic acid stains Syto 9 (one-photon excitation) and Syto 40 (two-photon excitation) at first did not show an extended heterotrophic bacterial community. These organisms only became visible as tiny spheres when the maximum-intensity projected series (shown in Fig. 9b) was examined layer by layer. Furthermore, two-photon imaging was used in combination with DAPI nucleic acid staining. The result showed the autofluorescence of the cyanobacteria within the bag as well as a DAPI signal inside the cyanobacterial cells (Figs. 8e and 9c). Due to the channel settings for recording the emission signals at 400 – 575 nm, the green autofluorescence of the sheath material is picked up in the same channel as the DAPI signal and therefore is imaged in the same color (green).

The application of lectins allowed imaging of glycoconjugates associated with the endolithic phototrophs (Fig. 9c). The lectin of *Aleuria aurantia* bound to matrix polymers in which the phototrophic cells were embedded. Interestingly, not all phototrophic cells, even of similar morphology, reacted with the lectin, indicating different cyanobacterial strains with matrix polymers of different lectin-binding properties (Fig. 9c). Isosurface presentation shows the 3-D arrangement of phototrophic cells within the glycoconjugates (Fig. 10).

Discussion

Bare dolomite rocks in the Piora Valley are ideal sites for the study of endolithic microbial systems. Phototrophic microorganisms easily penetrate porous dolomite and are observed as a distinct brown-gray to green endolithic band some millimeters below the rock surface when the surface is split off. Microbial life at these sites is limited by environmental factors such as desiccation and lack of water for long periods, a large span of fluctuating temperatures including freeze-thaw cycles, scarce nutrient availability, and variable high photosynthetic active radiation (PAR) intensity combined with a large UV-B (290-320 nm)

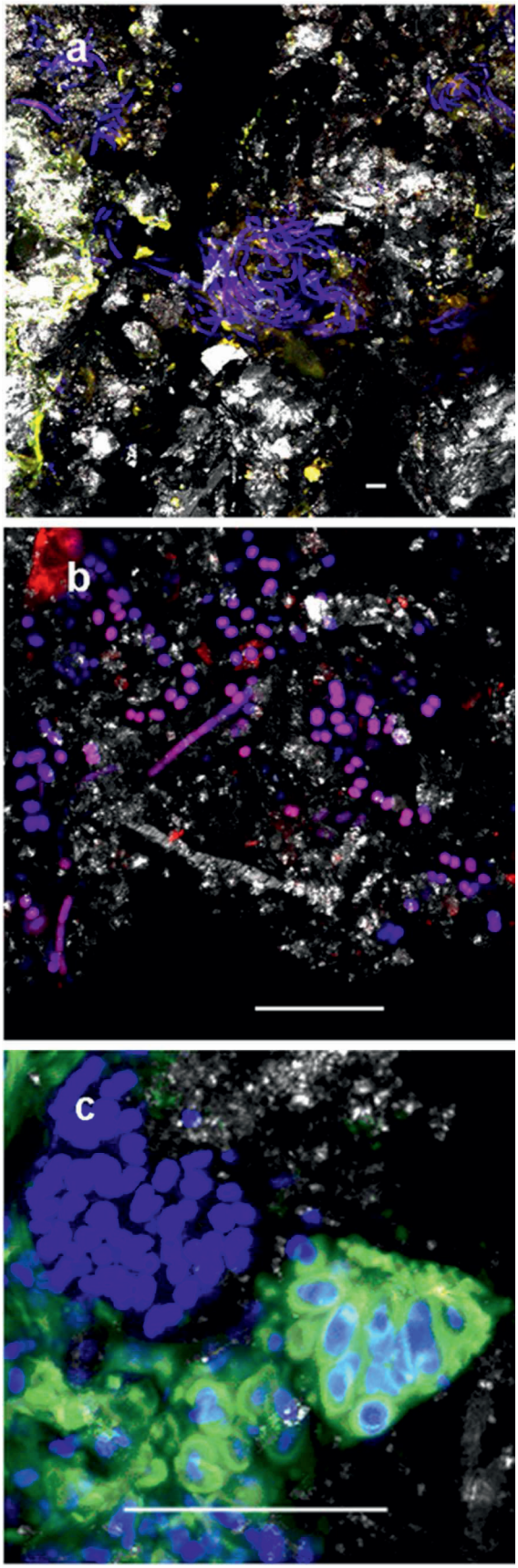

Figure 9. Maximum intensity projection of CLSM images from endolithic microbial community showing locations with different cell types and EPS glycoconjugates. Color allocation: reflection from minerals, white; colocalized autofluorescence of cyanobacteria in the red and far-red channel, pink/red/blue (bar=20 mm). (a) Overview showing mineral reflection and autofluorescence of a microcolony with bent filamentous phototrophic cells. (b) Location with autofluorescence of coccoid and straight filamentous phototrophic cells. Sample was also stained by a live nucleic-acid- specific stain. Color allocation of Syto9, green. (c) Phototrophic microcolony after lectin staining showing cell clusters with and without glycoconjugate production. Color allocation of lectin, green.

fraction at the rock surface [26]. Such conditions are similar to extreme environments found in the Arctic and Antarctic, where endolithic organisms form one of the few ecosystems [5, 6]. These environments have also been suggested as possible analogs for living conditions on Mars [20, 22].

Physical Properties of the Environment. Light penetration into the rock clearly depends on the angle incident to the surface (Fig. 2). When entering perpendicular to the surface, up to 5% of the surface intensity is still available for photosynthesis at 2 to 3 mm depth within the rock, which compares well with the depth at which the endolithic band is found at bright-light sites. In diffuse illumination, light intensity drops more rapidly with depth. Similar data have been reported by Diels [7], whereas light intensities for the Niagara cliffs

are more comparable to shadowed dolomite rock [21]. The phototrophic band in the dolomite rock has a clear inner boundary, suggesting that a specific environmental factor limits further penetration of the organisms into the rock. Jaag [19] suggested that the cyanobacteria are limited by the decreasing water availability with depth. The variable position of the cyanobacteria according to the mean light intensity (Fig. 2) rather suggests that the limiting factor is more likely the light intensity.

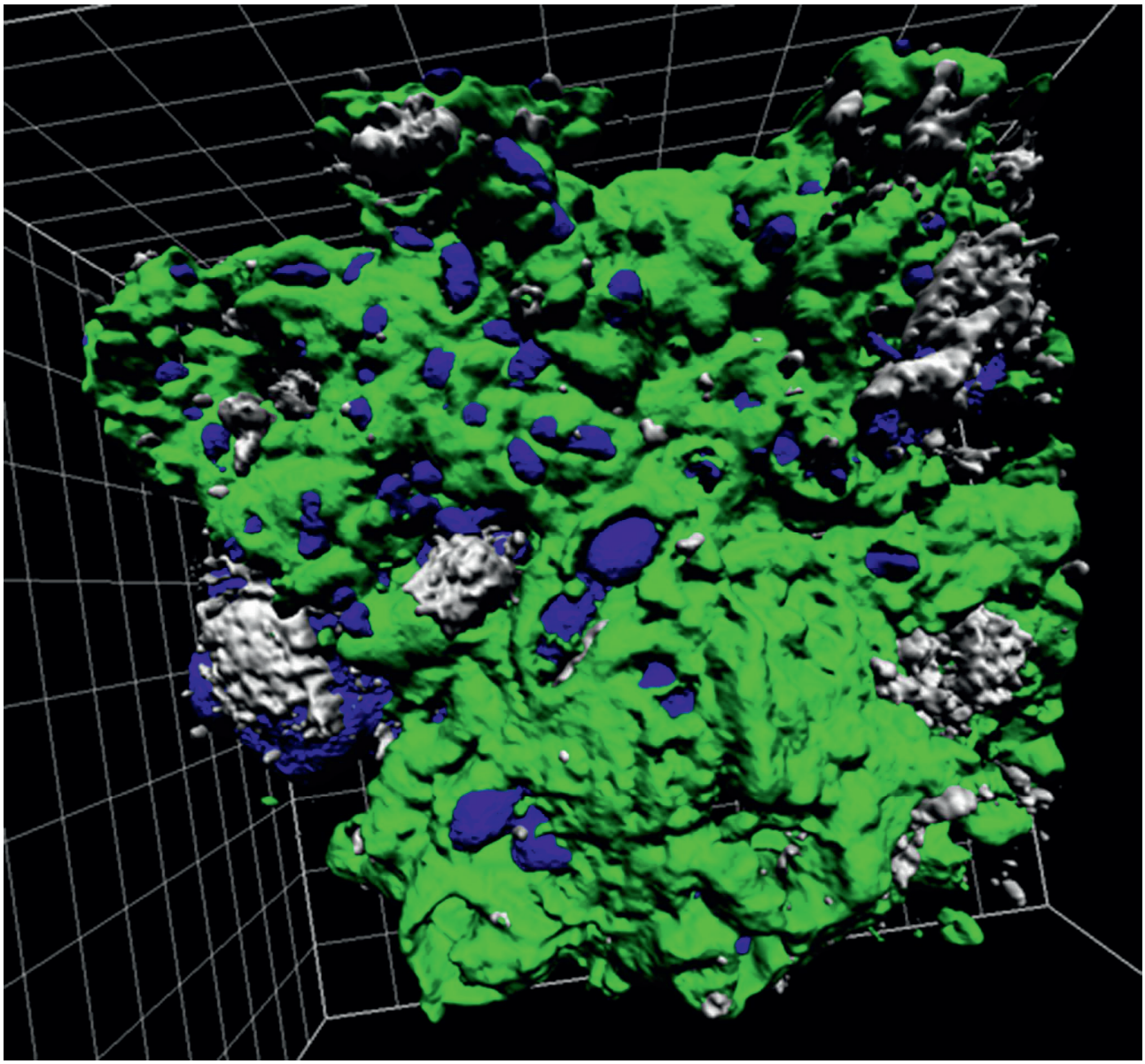

Figure 10. Three-channel isosurface presentation of an endolithic phototrophic microcolony with associated glycoconjugates. Most of the phototrophic cells (blue) attached to mineral surfaces (white) are deeply embedded in a glycoconjugate layer visualized by lectin staining (green) (grid size=20 mm).

Composition of the Community from Spectral Studies. From microscopy studies [e.g., 1, 5, 19, 30, 34, 38], it is obvious that the major primary producers in

endolithic biofilms are cyanobacteria. Cyanobacteria are known to be well adapted to conditions of high environmental stress [14, 17, 38] and thus are the prevalent primary producers in such conditions. The fundamental study of Jaag [19] on algal vegetation on bare rocks in Switzerland lists 210 species of mainly epi- but also endolithic phototrophic microorganisms, with 48.6% belonging to the cyanobacteria. This dominance is demonstrated in our samples in the reflection spectra (Fig. 3), which indicate the presence of oxygenic phototrophs containing Chl *a* and varying amounts of the accessory phycobilins. In some samples, a third pigment with an absorption maximum at about 720 nm (or 713 nm) was observed. The distinct shoulder at 720 nm (713 nm) observed at some low-light sites (Fig. 3b) indicates the presence of a probably new bacteriochlorophyll. Of the bacteriochlorophylls known, Bchl *e* and certain forms of Bchl *d* exhibit an absorption in this region [2]. This is supported by the spectrum of the methanolic extract of rock material and by selected spectra obtained after HPLC separation (Fig. 5). The peak in the raw extract at about 664 nm (Fig. 5a) is interpreted as the sum of Chl *a*, Chl *b*, and a bacteriochlorophyll type *d* or *e*. As the long-wavelength peaks of these pigments overlap, the exact pigment composition can only be determined after chromatographic separation. HPLC separation gave evidence for various unidentified carotenoids and several chlorophyll-like absorbing compounds. The dominance of Chl *a* (Fig. 5h) from the cyanobacteria had to be expected; Chl *b* (Fig. 5f) had about 20% absorption of Chl *a*. However, the absorption at 466 nm could also suggest the presence of a bacteriochlorophyll from green bacteria [11, 18]. In low concentration with an absorption strength of a few percent of Chl *a*, a so far undescribed chlorophyll-like pigment was present with absorption maxima of 440 nm and 706 nm (Fig. 5g). As the pigment observed by reflection spectroscopy absorbing *in vivo* at 720 nm was not frequently seen, the one given in Fig. 5g might be the same. The spectrum in Fig. 5e with a main band at 770 nm suggests the presence of a very small population of phototrophic bacteria containing Bchl *a* [18].

Phycobilins as markers of cyanobacteria were less dominant in samples showing the 720-nm absorption (Fig. 3b vs a). The dominance of oxygenic phototrophs was imaged in previous results from enrichment cultures with endolithic material as inoculum, where growth of the green algae *Stichococcus* and *Chlorella* spp. and of the cyanobacteria *Nostoc* and *Calothrix* spp. was observed [30]. Sequence data suggested a range of cyanobacteria, including relatives of *Leptolyngbya*, *Scytonema*, *Microcoleus*, *Chrooccidiopsis*, and *Anabaena*. Interestingly the most dominant genus in the studies of

Jaag [19], the coccoid *Gloeocapsa* (sanguinea and Kützingiana), was not detected by molecular means [30].

Localization of the Microbial Community by Spectroscopy. Spectroscopy allowed differentiation between eukaryotic algae and cyanobacteria based on the presence of phycobilins. A profile of the photosynthetic pigments across the endolithic band suggested that eukaryotic green algae are located closer to the surface, whereas the cyanobacteria are found in deeper zones (Fig. 4a), which was confirmed by quantitative image analysis of CLSM cross sections (Fig. 7). In cyanobacteria, the ratio between chlorophyll and the accessory phycobilins is indicative of chromatic adaptation. Indeed, the phycobilins increased in relation to the chlorophyll with depth (Fig. 4a). Similar observations were reported by Quesada *et al.* [28] in arctic samples.

Both trans-sections in Fig. 4 exhibited distinct peaks in the distribution of the pigments, indicating that the phototrophs in the endolithic layer are spatially ordered. Similar structures in endolithic systems were also described by Russel *et al.* [29] from the Antarctica, using FT-Raman spectroscopy. These authors point to differences observed between different sampling sites, which were some kilometers apart. In contrast, our measurements with optical tracing give evidence of a spatial microheterogeneity in the millimeter to centimeter range. Our data fit well with the model suggested by Pohl [27] for euendolithic biofilms in carbonate rocks with three major zones. On the outer side of the active layer of photobionts is a zone of partially dead and decaying cells; indeed, we often observed a reddish band [30] as well as absorption at 660 nm, which is indicative for free or denatured chlorophylls. According to Jaag [19], *Gloeocapsa* sp. were the most frequent cyanobacteria in rock, carrying a red or violet gelatinous envelope of variable thickness. In fact, a reflection spectrum of such "red" dolomite (data not shown) yielded a similar spectrum as given by Jaag [19].

Localization of the Microbial Community by Microscopic Methods. Laser scanning microscopy (LSM) is an excellent technique to visualize *in situ* microbial communities at interfaces and in complex matrices and is thus the tool of choice in biofilm research [24]. The technique was already employed for imaging phototrophic microbial biofilms and mats in various habitats [32, 36]. This approach can be taken a step further by using one-photon and two-photon LSM as a tool for imaging, differentiation, and quantification of phototrophic microorganisms within microbial biofilms [25]. It was also applied in the present study. The LSM results of Piora dolomite showed different

cyanobacterial coccoid morphotypes. Confocal images revealed predominantly coccoid organisms with bright phycobilin autofluorescence, this morphotype seems also to be typical for dry limestones, which only temporarily get wet [16]. Most cells were present in microcolonies; some of these were covered by a thick layer of glycoconjugates and embedded as densely packed microcolonies in a thin envelope. Surprisingly, the envelope became visible without staining due to its green autofluorescence. The microcolonies within the envelope had either the known colocalized cyanobacterial pink signal or a green autofluorescence similar to the one of the envelope. This green autofluorescence has not been seen in liquid cultures prepared from endolithic material (data not shown). Furthermore, the green autofluorescence from one-photon excitation (488 nm) was confirmed by two-photon excitation LSM (Figs. 8a-c compared to d and e). This finding remains unclear and needs more investigation. Staining with a fluorescent lectin reveals strong binding toward the glycoconjugates of some cyanobacterial strains (Figs. 9e, f). Yet, in order to stain a greater proportion of the cyanobacterial glycoconjugates, a screening for specific lectins, as suggested by Staudt *et al.* [33], would be necessary. In Fig. 10, the 3- D structure of the three components becomes visualized, the cells in blue, the surrounding EPS in green, and the rock material in white-gray. Cells are embedded in the polymer matrix, which fully fills out the pore space.

Physiological Adaptation to the Environment . Cyanobacteria as evolutionarily ancient organisms may contain UV-shielding pigments, scytonemins, and mycosporine-like amino acids (MAAs) [8, 13]. One compound after HPLC separation (Fig. 5b) is indicative for scytonemins [3, 12]; this peak has a large absorption of about twice the value of Chl *a*. The biosynthesis of such compounds is not only induced by UV illumination, but other harsh environmental factors, such as temperature and osmotic or photooxidative stress, may also act synergistically and enhance the accumulation of UV-protecting compounds [4]. Garcia-Pichel and Castenholz [13] found scytonemin extracellularly in the sheaths of colonies and cells. After irradiation with UV-B, Nostoc produced MAAs absorbing at 335 nm [9]. These substances exhibit fluorescence with an emission in the blue region at 436 nm [31]. However, as these compounds absorb in the UV they are not detected by reflection spectroscopy but possibly with UV or two-photon excitation LSM (Figs. 8d, e).

Interestingly, irradiation of *Nostoc* with UV-B not only stimulated the formation of MAAs but also increased the dry weight of cells two- to threefold due to glycan formation, which was deposited as sheath material [9]. The large volume of EPS relative to that of the

enclosed cells in the endolithic band may indicate the stress situation in this dolomite rock environment.

Heterogeneity of the Distribution of the Organisms within the Endolithic Band. Although observed by the naked eye the endolithic band looks homogeneous and continuously following a line a few millimeters below the rock surface, a minor magnification (Fig. 1) demonstrates that the dimensions of the band are variable and the distribution of the cells within the band very heterogeneous. This is especially clear in the CLSM cross section (Fig. 7) with local accumulations of packed cells. Deeper inside the rock, single cells still exist. This heterogeneity is not seen in the reflection spectra cross sections, as this technique has not the necessary spatial resolution and averages over distances of about 0.5 mm. Still, large differences in pigment reflection intensity were found between different sites of the same sample. Furthermore, the depth of the band and its size show a broad variation, e.g., at a high-light site 2.27 $\pm$ 0.82 mm (SD) as mean depth, with extremes of 0.5 mm and 4.8 mm. This can hardly be explained by the light regime. Weathered dolomite is heterogeneous concerning its porosity, which controls the water availability of the cells. Unfortunately, microelectrodes cannot solve such questions in rock material. Organisms with an absorption at 720 nm are restricted to shaded sites and there located toward the inside of the band, they seem to be less stress tolerant toward light and temperature.

Conclusion

The study using different methods demonstrated a heterogeneous distribution of the cells and the specific pigments as markers of phototrophic organisms in the endolithic band. It also suggested multiple survival strategies of the endolithic population. Besides being buried within the rock, the cells protect themselves from intense light and UV by producing UV-shielding pigments that become deposited in the exopolymers and may be the cause of the green autofluorescent spheres and envelopes surrounding the cyanobacterial micro-colonies (Fig. 8). The massive extracellular polymeric substances in the form of glycoconjugates may also protect the cells from dehydration (Figs. 9c and 10), adsorb water and nutrients, and recycle the latter within the living endolithic microbial community. Both functions make sure that the cyanobacterial cells survive extended periods of drought. It will be of high interest to obtain information on the heterotrophic community accompanying the primary producers.

Acknowledgments

We thank Drs. F. Schanz and M. Wiggli for fruitful discussions, the help concerning statistical calculations, and treatment of the *in vivo* spectra. We are much indebted to Dr. B. Wise for his help in clarifying the English text and Dr. J. Blom for the HPLC analyses. The excellent technical assistance of U. Kuhlicke (LSM) and U. Jauch (SEM) is appreciated.

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Published in: *Microbial Ecology* (2009) **58**:290-306

(3.) Molecular characterization of an endolithic microbial community in dolomite rock in the central Alps (Switzerland)

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DOI 10.1007/s00248-008-9483-7
http://springerlink.metapress.com/content/1826014w23148042/?p=6e0337667ae942aa9a2e5ea520c9a71f&pi=0

Abbreviations

Na_2EDTA: ethylenediaminetetraacetic acid disodium salt dehydrate; Tris-HCl: 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride; Tris base: 2-amino-2-hydroxymethyl-propane-1,3-diol; SDS: sodium dodecyl sulfate; TAE buffer: 40 mM Tris base, 20 mM acetic acid, 1 mM Na_2EDTA (pH 8.0); RFLP: Restriction fragment length polymorphism; PBS: phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4; DAPI: 4',6-diamidino-2-phenylindole; BSA: bovine serum albumin. SSU rRNA gene: small subunit of the ribosomal ribonucleic acid gene (16S/18S rRNA)

Abstract

Endolithic microorganisms colonize the pores in exposed dolomite rocks in the Piora Valley in the Swiss Alps. They appear as distinct grayish-green bands about 1-8 mm below the rock surface. Based on environmental small subunit ribosomal RNA gene sequences, a diverse community driven by photosynthesis has been found. *Cyanobacteria* (57 clones), especially the genus *Leptolyngbya*, build the functional basis for an endolithic community which contains a wide spectrum of so far not characterized species of chemotrophic *Bacteria* (64 clones) with mainly *Actinobacteria*, *Alpha-Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*, as well as a cluster within the *Chloroflexaceae*. Furthermore, a cluster within the *Crenarchaeotes* (40 clones) has been detected. Although the eukaryotic diversity was outside the scope of the study, an amoeba (39 clones), and several green algae (51 clones) have been observed. We conclude that the bacterial diversity in this endolithic habitat, especially of chemotrophic, nonpigmented organisms, is considerable and that *Archaea* are present as well.

Introduction

Microorganisms inhabiting rock were first observed and described 100 years ago [19, 45, 79], nevertheless, except for cyanobacteria, little is known about the community composition and the biodiversity of these microbial ecosystems. They are typical for hot and cold arid environments where in the pores of the rock, they are partially sheltered from a number of physical stresses such as solar radiation, heat, cold, or desiccation. Various organisms settle on the surface and invade pores and cracks. Within the rock, they form a structured biofilm, a clearly defined organismic layer or band a few millimeters below the surface [15-17, 24-26, 35, 42, 44, 58, 65, 88-90]. Contrary to submerged biofilms, endolithic biofilms are patchier due to local inhomogeneities of rock structures and environments [42]. These communities contain bacteria, fungi, and eukaryotic microalgae [81, 82]. They form complex physiological networks tied to solid particles by extracellular polymeric substances (EPS). The synthesis of these polymers is controlled by different environmental stress factors (e.g. [77]). The organismic composition is governed by the hostile environment. Water is only periodically available in the form of rain, dew, or just atmospheric humidity. Therefore, EPS are most important for the endolithic population as they retain water and act as osmoprotectant and nutrient reservoirs. In the Alps, main nutrients are scarce, and the daily and seasonal temperatures oscillate widely. At high altitudes, the sunlight with a strong part in the UV is a further life threatening factor [95]. Habitats with such fluctuating environmental conditions pose a strong challenge to organisms, and life there may reach its limits at least in certain periods.

Endolithic microorganisms have gained interest in the past decades for several reasons: e.g., as possible analogs of extraterrestrial life, such as life on Mars [2, 24, 40, 43, 46, 59, 63, 73, 94, 95], for the study of the mechanisms of adaptation to extreme and hostile conditions [30, 36,

87, 96], to study the processes of weathering and mineral dissolution [12, 91] or for phylogenetic reasons [17, 29, 65, 86, 88, 89].

Endolithic microbial communities are found worldwide in dry and aquatic environments. The ones studied and described came from cliffs of the Niagara escarpment [32, 56, 57] from streams in the UK [68] and from gypsum cliffs in Nova Scotia [28]. They are found in hot and arid desert environments [5-7, 23], in travertine in Turkey [69], in arctic and antarctic locations [2, 26, 27, 44, 74, 93], in mountainous regions [42, 65, 88, 89], and in the marine littorals [92]. Most investigations have been based on traditional techniques, mainly light and electron microscopy, and on cultures. They have usually been focused on pigmented microorganisms, oxygenic phototrophs such as green algae and cyanobacteria as well as filaments of fungi as partners of lichen symbiosis. Cyanobacteria are important in the early stages of primary succession processes in soils, especially because many species are able to fix dinitrogen [47]. However, it must be assumed that a variety of heterotrophic organisms will rapidly follow the phototrophs after their invasion. So far, molecular methods have hardly been used. They have even been thought to be useless in studying endoliths [93]. However, molecular techniques are now successfully applied to characterize endolithic communities such as the cyanobacterial population in the dolomite rocks in Switzerland [82], the endolithic community in the McMurdo Dry Valleys in the Antarctica [17], or the microbial population in rocks of the Rocky Mountains [29, 65, 89].

The objective of the present study is to describe the broad genetic diversity of the endolithic bacterial populations present in the dolomite formations of the Swiss Alps by culture independent molecular methods. Dolomite rocks ($CaMg(CO_3)_2$) in the Piora Valley in southern Switzerland are often bare of vegetation and exposed to hostile conditions. Such weathered rocks harbor chasmoendolithic and cryptoendolithic (definitions, see [34]) phototrophic and heterotrophic microbial communities which become easily visible as grayish-green bands some millimeters below the surface. This hidden microbial ecosystem was first characterized by Diels [19] in the Italian Dolomites and has been studied in Piora dolomite by molecular [82], spectroscopical, and optical techniques [42]. At a depth of 2 to 8 mm, the phototrophic microorganisms still receive enough photosynthetic active radiation while they are protected from excessive sunlight with a high fraction in the UV range [42]. As most organisms of environmental samples cannot be cultured by standard methods yet, a description of the microbial diversity of this special microbial ecosystem has been obtained by sequence analyses of polymerase chain reaction (PCR) amplified fragments of the small subunit of the ribosomal ribonucleic acid gene (SSU rRNA gene). The knowledge of the composition of the microbial community will help to better understand the biogeochemical processes that occur in these habitats. Preliminary results have been presented earlier [41, 81].

Materials and Methods

Sampling Site

Dolomite rock material was collected in the Piora Valley in the southern part of the Swiss Alps at an elevation of 1965 m above sea level in summer 2001 and 2003. The coordinates of the specific sampling site are 46°32′51″ N, 8°43′05″ E. Details of the site are given by Sigler et al. [82] and Horath et al. [42]. The geology of the Piora Valley, oriented east- west, is characterized by a dolomite trough, a few hundred meters wide, surrounded by crystalline rock formations. Due to erosion by wind and water, the dolomite is often exposed to the atmosphere, forming white cliffs. Such sites are sparsely covered with black epilithic cyanobacteria and lichens. Especially in slightly weathered dolomite, endolithic microorganisms are easily observed when the surface layer is removed. They form a grayish-green layer about 1-8 mm below the rock surface. Rock pieces of some millimeters or centimeters in size were cut off from the surface with an ethanol-flamed chisel and hammer, and samples with visible endolithic bands were kept in Falcon tubes in the dark at 4℃ until DNA extraction in the laboratory.

DNA Extraction

DNA extraction was performed as described by Sigler et al. [82]. In brief, 0.5 to 0.6 g of rock samples of the green layer was scratched into a sterile empty Petri dish with sterilized tools, then put into 2-ml sterile microfuge tubes containing 1.0 ml of extraction buffer (50 mM NaCl, 50 mM ethylene diamine tetra acetic acid disodium salt dihydrate (EDTA; Fluka 03685), 50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (TRIS-HCl; Fluka 93363) and 5% sodium dodecyl sulfate (SDS; Fluka 71729), final pH 8), 0.5 g glass beads (0.1 and 0.5 mm in diameter) and eventually 0.5 ml of a phenol-chloroform-isoamylalcohol- mixture (v/v/v=49.5/49.5/1, Fluka 77618). The tubes were sealed with Parafilm®, shaken in a bead beater ("FastPrep®", BIO 101, La Jolla, CA, USA) at 5.5 m s^{-1} for 30 s and centrifuged for 4 min at 10'000×*g*. Nucleic acids were isolated from the watery supernatant by standard phenol/chloroform extraction and ethanol precipitation [75]. The dry DNA pellet was redissolved in 50 µl distilled autoclaved water.

PCR Amplification of SSU rRNA Genes

The small subunit rRNA gene was amplified from genomic DNA by PCR with several pairs of primers (see Table 1). PCR was performed in 200-µl thin-walled tubes on a "Progene" or a "Genius" thermocycler respectively (Techne LTD, Duxford Cambridge, U.K) in a volume of 25 µl. The reaction mixture contained (final concentrations):

Table 1 List of clone library names, sequences of primers, and numbers of different clones obtained (3% sequence difference level)

Library Name	Primer	Primer Sequence (5' to 3')	Reference	Obtained Products	Total Clones	Sequenced Clones	Different Clones
Dolo	27f	AGA GTT TGA TCM TGG CTC AG	[20, 50]	*Bacteria* / Chloroplasts	36	30	22
	1524r	AAG GAG GTG ATC CAR CCG	[50] Slightly modified				
DoAr	8aF	TCY GGT TGA TCC TSC C	[11] Slightly modified	*Euamoeba* sp.	39	1	1
	1517r	ATC CAG CCG CAG RTT C	this paper				
ud	536f	CAG CMG CCG CGG TAA TWC	[49]	*Bacteria* / *Crenarchaea*	35	16	10
	1392r	ACG GGC GGT GTG TRC	[49]				
DA	8aF	TCY GGT TGA TCC TSC C	[11] Slightly modified	*Chlorella* sp. (18S)	16	8	4
	1512uR	ACG GHT ACC TTG TTA CGA CTT	[50] Slightly modified 1492r				
DOS	89Fb	ACG GCT CAG TAA CRC	[10]	*Crenarchaea*	38	10	3
	915R	GTG CTC CCC CGC CAA TTC CT	[85]				
DOL	8aF	TCY GGT TGA TCC TSC C	[11] Slightly modified	*Bryophyta* (18S)	28	5	1
	1512uR	ACG GHT ACC TTG TTA CGA CTT	[50] Slightly modified				
DoCY	CYA359F	GGG GAA TTT TCC GCA ATG GG	[66]	*Cyanobacteria*	23	9	6
	CYA1342R	GAC CTG CAA TTA CTA GCG	[78]				
Docu	CYA359F	GGG GAA TTT TCC GCA ATG GG	[66]	*Cyanobacteria*	36	17	6
	CYA1342R	GAC CTG CAA TTA CTA GCG	[78]	and Chloroplasts			

	Sequencing Primers (5' to 3')	Reference
M13 forward	GTA AAA CGA CGG CCA G	[60]
M13 reverse	CAG GAA ACA GCT ATG AC	[60]
519r	GWA TTA CCG CGG CKG CTG	[49]
536f	CAG CMG CCG CGG TAA TWC	[49]
1099r	GGG TTG CGC TCG TTR C	[50] Slightly modified
1114f	GYA ACG AGC GCA ACC C	[50] Slightly modified

The appropriate *Taq* buffer (1×), 1.5-2.0 mM $MgCl_2$, 0.1 mg ml^{-1} bovine serum albumine, 0.2 mM dNTP's, 200 nM forward primer, 200 nM reverse primer, 40-100 U ml^{-1} *Taq* Polymerase (Sigma, Promega, Invitrogen, or Pharmacia), and approximately 50-100 ng template DNA. PCR was run under the following conditions: initial denaturation at 94℃ for 2 min, 10 cycles of 94℃ for 20 s, 60℃-0.5℃/cycle for 30 s, 72℃ for 60 to 90 s depe nding on the length of the product, 20 cycles of 94℃ for 20 s, 50℃ to 58℃ for 30 s, depending on the annealing temperature of the primers, 72℃ for 60 to 90 s. The products were checked on a 1% agarose gel in 0.5× TAE buffer [1×=40 mM Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol), 20 mM glacial acetic acid, 1 mM Na_2EDTA of pH 8.0].

Cloning

PCR-amplified products were cloned without purification with the TOPO TA cloning kit (Invitrogen, K4500-01) as specified by the manufacturer's manual.

Restriction Fragment Length Polymorphism (RFLP)

After plasmid DNA mini preparation with alkaline lysis [75] and the reamplification of the SSU rRNA gene with M13 primers, restriction was carried out with *Hin*f I and *Hae* III and the fragments analyzed on a Spreadex® EL 800 Wide Mini S-50 gel (Elchrom Scientific) run at 55℃ for 1 h at 10 V cm^{-1}. The gels were stained with 10'000 times diluted 1% (w/v) ethidium bromide and viewed with 302 nm UV illumination.

DNA Sequencing

Reamplified plasmid inserts were purified by filtration (Amicon Microcon YM-100 filter, Millipore Corporation, Bedford, MA, USA), and 100 to 180 ng DNA (dissolved in 1 µl H_2O) were used for sequencing-PCR using 0.8 µl BigDye® Terminator v3.1 (Applied Biosystems), 1.5 µl sequencing buffer (5×), 6.8 µl of H_2O Milli Q, and 0.25 µl (5 µM) of one of the sequencing primers listed in Table 1. Before the automated loading into the polymers on the 48-capillary sequencer (Applied Biosystems 3730 DNA Analyzer), the PCR products were purified by centrifugation through Sephadex G50 (Amersham Pharmacia). The raw sequences were aligned and combined using the Gene Codes Sequencher software (www.genecodes.com).

Nucleotide Sequence Accession Numbers

The SSU rRNA gene sequences found have been deposited at the DNA Data Bank of Japan and can be retrieved under the accession numbers AB257629 to AB257698 and AB334273 to AB334298.

Phylogenetic Tools

Rarefaction curves were generated with the program "Analytic Rarefaction 1.3" provided by Steven M. Holland at "http://www.uga.edu/~strata/software/Software.html". The newly obtained SSU rRNA gene sequences were compared with known sequences in the NCBI database (Genbank) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) by the use of the Basic Local Alignment Search Tool (BLAST) [1] to determine their approximate phylogenetic affiliation.

The EMBOSS Pairwise Alignment Tool at "http://www.ebi. ac.uk/emboss/align/" provided by the European Bioinformatics Institute was used to compare single sequences in the following mode: "Method: water"; "Gap Open: 10.0"; "Gap Extend: 10.0"; "Molecule: DNA"; "Matrix: DNAfull".

The new SSU rRNA gene sequences were further added to the rRNA gene sequence database of the Technical University of Munich (ssu_jan04_corr_opt.arb, release February 2005) by the use of the program package ARB ([54], http://www.arb-home.de). The integrated tool ARB_ALIGN was used for automatic sequence alignment, which was then checked with a critical eye according to the secondary structure of the rRNA molecule, and corrected. If missing, the latest best fitting sequences found by NCBI- BLAST were added to the ARB database.

The final phylogenetic trees were derived from the basic phylogenetic tree of about 51’000 SSU rRNA gene sequences after adding the new sequences with appropriate filters, and the "Maximum Parsimony Method." Bootstrap values were calculated from the sequences used in the final trees by using the "Phylip Parsimony Method", integrated in ARB, compressing vertical gaps, running 100 bootstrap samples.

In order to plot a phylogenetic tree, many different algorithms are available today, which all lead to acceptable results if they are based on a proper sequence alignment [52]. Therefore, emphasis has been put on an accurate alignment. The trees presented are copies of the largest tree, namely "tree_1000_jan05" in the ARB data- base "ssu_jan04_corr_opt.arb". After adding the new sequences to the existing tree containing more than 50,000 single SSU rRNA sequences, the new trees have been reduced to a convenient size for illustration. Bootstrap values have been calculated although they are not considered to be very important, since these values can be shifted by omitting closely branching sequences before calculation (Eichenberger, Ch., personal communication).

Bootstrapping has been introduced to provide confidence intervals in phylogenetic calculations [13, 21], because calculated trees are never fully true and require flexible interpretations. When using Maximum Parsimony, Distance Matrix (Neighbor Joining), or Maximum Likelihood, the result should not be overestimated because its variation among different methods is a negligible indicator of the confidence interval [21]. Furthermore, the order of adding sequences to a calculation has an effect on the tree topology [e.g., 53]. Thus removing and readding complete

groups to a tree may rearrange its branching. In our case, it improved the congruence of the results of ARB and NCBI.

Results

In a previous study, we investigated endolithic bacterial communities in exposed weathered dolomite rocks by confocal laser scanning microscopy, pigment analysis, and reflectance spectroscopy [42]. Communities depending on photosynthesis usually harbor a sum of heterotrophic organisms which feed on exudates and lysed cells. As it is hardly possible to characterize the diversity of environmental microorganisms by cultivating them, we analyzed the endolithic heterotrophic community by cloning and sequencing their SSU rRNA genes.

SSU rRNA Gene Clone Libraries

Isolation of DNA from fine powdered rock material posed some difficulties as DNA tended to stick to and precipitate with the inorganic rock debris. Suitable amounts of DNA were obtained following the procedure of Sigler et al. [82]. To evaluate the diversity of the prokaryotic endolithic community, eight independent clone libraries with different combinations of universal and phylogenetic group-specific oligonucleotide primers were constructed, including two libraries with specific cyanobacterial primers (Table 1). In total, 254 clones were analyzed by restriction fragment length polymorphism (RFLP), 96 of which were sequenced. Assuming a threshold of a minimal 3% sequence difference between species [84], 53 sequences fell into distinctly related groups. From these 53 phylotypes, 45 belong to *Bacteria* (including three chloroplasts of two green algae and a moss), three to *Archaea*, and five to *Eukarya* (Table 2). Scanning the graphic alignment of the NCBI-BLAST analysis of the new sequences, no chimeras have been detected ([1], http://www.ncbi.nlm.nih.gov/).

A wide diversity was found in the clone libraries obtained with the bacterial primer pair 27f/1524r and the "universal" primer pair 536f/1392r. In the bacterial library, 22 out of 36, and in the "universal" library, nine out of 35 clones were different. The other primer pairs resulted in less diverse libraries. As an extreme, primer pairs 8aF/1517r (DoAr) and 8aF/1512uR (DOL) yielded 39 and 28 RFLP-identical clones, respectively (Table 2). Primer 1517r (Table 1) was originally designed to increase the number of *Archaea* clones but resulted in the detection of a so far unknown 18S rRNA gene sequence fragment closely related to *Saccamoeba limax* (99.4%, clone DoAr09).

Rarefaction curves for all the eight clone libraries are shown in Fig. 1. The shapes of the curves "Dolo" and "ud" indicate that further sampling would increase the number of operational

taxonomic units (OTUs, 3% difference level). In contrast, the other graphs, except for the summarized data, level off rapidly, a phenomenon for discussion.

A quarter of the obtained bacterial sequences (64 out of a total of 251 clones) originated from phototrophic oxygenic organisms. *Cyanobacteria* were numerous with 11 phylotypes, chloroplasts of green algae (Dolo-01, Dolo-34) or of bryophytes (Docu-30) with three different phylotypes (seven clones). Among the heterotrophic species, the representatives of the phylum *Actinobacteria* were the most numerous (15 clones, seven phylotypes), followed by *Alpha Proteobacteria* (14 clones, ten phylotypes), and *Bacteroidetes* (12 clones, two phylotypes). *Acidobacteria* (seven clones, two phylotypes), *Gamma Proteobacteria* (five clones, one phylotype), and *Gemmatimonadetes* (two clones, two phylotypes) were less frequent. Only one clone was found in each of the proposed divisions TM6 and TM7, as well as in the phylum *Planctomycetes* (Fig. 2 and Table 2). The green nonsulfur phototrophic bacteria group of the *Chloroflexi* yielded six clones (four phylotypes). The sum of bacterial phyla found in the dolomite of the Piora Valley covers ten of approximately 75 bacterial phyla known or postulated so far [51, 72, 76]. All the archaeal sequences found fell into the group of uncultured *Crenarchaeotes* (Table 2). Eukaryotic 18S rRNA gene sequences have been found in groups related to *Euamoebida*, *Bryophyta*, and *Chlorophyta* (83 out of a total of 251 clones, five phylotypes; Table 2). The phylogenetic trees give an overview of the distribution of the newly detected SSU rRNA gene sequences in the domains of *Bacteria*, *Archaea*, and *Eukarya* (Figs. 3a, b, 4, and 5).

Within all sequences analyzed, the percentages of sequence identity with SSU rRNA gene sequences available at GenBank (http://www.ncbi.nlm.nih.gov/) range between 85.2% and 99.7%. Clone "DOS_02", on a length of 791 bp, was even 99.9% identical with the uncultured archaeon clone HL17 (AJ608203) in loam from a bank of the river Waal in the Netherlands, while clone "Dolo-07", on a length of 1425 bp, shows only an 83.8% similarity with the uncultured Chloroflexus clone pItb-vmat-61 (AB294962) from a microbial mat in a shallow submarine hot spring in Japan (Table 2). For some sequences, ARB or "EMBOSS Pairwise Alignment Algorithms" have found different closest relatives as compared to NCBI-BLAST, but then often with smaller sequence coverage. Among the 45 different bacterial phylotypes, 18 (40%) were less than 95% identical to the closest 16S rRNA gene in the nucleotide sequence database, 14 phylotypes (31%) were in the range between 95% and 97% sequence identity, showing genus level relation [84], while 13 phylotypes (29%) were within the species level (more than 97% sequence identity).

Table 2. Phylogenetic affinities of SSU rRNA gene sequences obtained from dolomite in the Piora Valley, Central Alps.

Clone	Frequency [a]	Phylogenetic Affiliation	Closest NCBI-BLAST Match (Accession No.)	% Identity	Accession No.
Dolo-26	1/36	*Acidobacteria*	Uncultured bacterium clone Amb_16S_1159 (EF018708)	96.8	AB257649
ud01	6/35	*Acidobacteria*	Uncultured bacterium clone Elev_16S_1031 (EF019528)	98.8	AB257683
ud02	6/35	*Actinobacteria*	Bacterium Ellin504 (AY960767)	96.4	AB257684
Dolo-16	1/36	*Actinobacteria*	*Goodfellowia coeruleoviolacea*, strain NRRL B-24058 (DQ093349)	94.1	AB257641
Dolo-39	1/36	*Actinobacteria*	*Micrococcineae* strain Ellin124 (AF408966)	93.9	AB257657
ud31	3/35	*Actinobacteria*	Uncultured actinobacterium clone FBP460 (AY250884)	99.1	AB257697
Dolo-10	1/36	*Actinobacteria*	Uncultured bacterium AT425_EubY10 (AY053479)	91.8	AB257636
ud17	2/35	*Actinobacteria*	Uncultured bacterium clone C-F-15 (AF443586)	94.6	AB257690
ud19	1/35	*Actinobacteria*	Uncultured organism clone DLE037 (EF127609)	92.7	AB257692
Dolo-28	2/36	*Alpha proteobacteria*	*Brevundimonas variabilis* (AJ227783)	98.8	AB257650
Dolo-09	1/36	*Alpha proteobacteria*	Marine alpha proteobacterium strain V4.MO.17 (AJ508754)	94.9	AB257635
Dolo-08	1/36	*Alpha proteobacteria*	*Sphingomonas asaccharolytica*, strain IFO 15499-T (Y09639)	96.7	AB257634
Dolo-14	1/36	*Alpha proteobacteria*	*Sphingomonas asaccharolytica,* strain IFO 15499-T (Y09639)	97.4	AB257639
Dolo-04	1/36	*Alpha proteobacteria*	Uncultured alpha proteobacterium clone OS-C38 (EF612400)	95.6	AB257630
Dolo-11	1/36	*Alpha proteobacteria*	Uncultured bacterium clone "Hot Creek 25" (AY168723)	91.7	AB257637
Dolo-24	1/36	*Alpha proteobacteria*	Uncultured bacterium clone JSC8-E1 (DQ532238)	97.9	AB257648
Dolo-22	3/36	*Alpha proteobacteria*	Uncultured proteobacterium 59H11 (AF245037)	98.5	AB257646
Dolo-05	2/36	*Alpha proteobacteria*	Uncultured soil bacterium clone PK_XIII (EF540444)	97.0	AB257631
Dolo-32	1/36	*Alpha proteobacteria*	Uncultured soil bacterium clone PK_XIII (EF540444)	93.0	AB257653
ud04	7/35	*Bacteroidetes*	Uncultured Bacteroidetes bacterium clone J35E6 (DQ365993)	96.5	AB257685
ud10	5/35	*Bacteroidetes*	Uncultured soil bacterium clone M52_Pitesti (DQ378268)	98.2	AB257688
Dolo-06	5/36	*Gamma proteobacteria*	Xanthomonas-like sp. V4.BO.41 (AJ244722)	97.3	AB257632
Dolo-19	1/36	*Gemmatimonadetes*	Uncultured bacterium clone 5-31 (DQ833469)	90.4	AB257644
Dolo-18	1/36	*Gemmatimonadetes*	Uncultured Gemmatimonadetes clone Skagen138 (DQ640715)	93.4	AB257643
Dolo-21	1/36	*Planctomycetes*	*Planctomyces* sp. (strain: Schlesner 658) (X81954)	96.7	AB257645
Dolo-31	1/36	TM6	Uncultured bacterium clone Ebpr8 (AF255643)	93.5	AB257652
ud08	1/35	TM7	Uncultured candidate division TM7 bacterium clone 71 (AF513102)	92.1	AB257687
DoCY-44	4/23	Cyanobacteria	Gloeobacter violaceus PCC 7421 (BA000045) / (AP006573)	95.7	AB334275
Docu-04	3/36	Cyanobacteria	Leptolyngbya frigida ANT.LH52.2 (AY493575)	95.0	AB334284
Docu-01	22/36	Cyanobacteria	Leptolyngbya sp. CENA 112 (EF088337)	96.9	AB334282
Docu-19	4/36	Cyanobacteria	Leptolyngbya sp. CNP1-B3-C9 (AY239600)	94.2	AB334292

Docu-28	3/36	Cyanobacteria	Leptolyngbya sp. Greenland_7 (DQ431002)	95.1	AB334294
DoCY-46	4/23	Cyanobacteria	Nostoc sp. 'Pannaria aff. leproloma cyanobiont' (EF174228)	98.9	AB334277
DoCY-45	1/23	Cyanobacteria	Uncultured cyanobacterium clone 100M1_F2 (DQ514011)	93.2	AB334276
DoCY-55	1/23	Cyanobacteria	Uncultured cyanobacterium clone 100M1_F2 (DQ514011)	96.7	AB334280
DoCY-42	8/23	Cyanobacteria	Uncultured cyanobacterium clone HAVOmat106 (EF032780)	94.0	AB334274
Docu-24	2/36	Cyanobacteria	Uncultured cyanobacterium clone HAVOmat31 (EF032786)	94.1	AB334293
DoCY-39	5/23	Cyanobacteria	Uncultured Gloeobacter sp. clone HAVOmat17 (EF032784)	95.8	AB334273
Dolo-23	1/36	uncultured Chloroflexi	Uncultured Chloroflexi bacterium clone AKYH1480 (AY922118)	96.0	AB257647
ud07	2/35	uncultured Chloroflexi	Uncultured Chloroflexi bacterium clone AKYH1521 (AY922125)	99.7	AB257686
Dolo-07	2/36	uncultured Chloroflexi	Uncultured Chloroflexus clone pltb-vmat-61 (AB294962)	83.8	AB257633
Dolo-17	1/36	uncultured Chloroflexi	Uncultured Chloroflexus clone pltb-vmat-61 (AB294962)	85.2	AB257642
DOS_21	7/38	Crenarchaeota	Uncultured archaeon clone DRV-A006 (AY923076)	98.2	AB257680
DOS_02	21/38	Crenarchaeota	Uncultured archaeon clone HL17 (AJ608203)	99.9	AB257674
DOS_05	10/38	Crenarchaeota	Uncultured archaeon clone JFJ-WS-Arch07 (AJ867731)	99.6	AB257676
ud14	2/35	Crenarchaeota	unidentified archaeon SCA1150 (U62812)	99.4	AB257689
DOL_01	28/28	Bryophyta	Blindia acuta (AF023681)	99.7	AB257668
DA-01	7/16	Chlorophyta	Pseudomuriella sp. Itas 9/21 14-1d (AY195974)	92.3	AB257659
DA-04	6/16	Chlorophyta	Stichococcus bacillaris K4-4 (AB055866)	98.6	AB257661
DA-12	3/16	Chlorophyta	Uncultured Dunaliellaceae clone Amb_18S_930 (EF023670)	94.6	AB257663
Docu-30	2/36	Chloroplast	Chloroplast of Hymenostylium recurvirostre (DQ629553)	99.7	AB334295
Dolo-34	1/36	Chloroplast	Uncultured chlorophyte clone FQSS008 (EF522228)	96.9	AB257654
Dolo-01	4/36	Chloroplast	Uncultured chlorophyte clone FQSS008 (EF522228)	97.5	AB257629
DoAr-09	39/39	Euamoebida	Saccamoeba limax (AF293903)	99.5	AB257667

[a] The frequency of the clones is given as the number of clones of one sort of phylotype divided by the total number of clones in that library.

Bacterial Community

The quantitative distribution of the different mostly heterotrophic phylotypes in the bacterial clone libraries "ud" and "Dolo" (excluding chloroplasts and the specific cyanobacterial libraries "Docu" and "DoCY") is diagrammed in Fig. 2. There are four predominant groups accounting for more than 80% of 64 clones: the *Actinobacteria* together with *Proteobacteria* (*alpha* and *gamma*), *Bacteroidetes*, and *Acidobacteria* are the most numerous. Looking separately at individual bacterial phylotypes, the five clones ud01, ud02, ud04, ud10, and Dolo-06 are the most numerous ones, all in all accounting for 45% of the non-oxigenic "ud" and "Dolo" clones. Based on NCBI-BLAST [1], these phylotypes represent *Bacteroidetes* (ud04=10.9%,

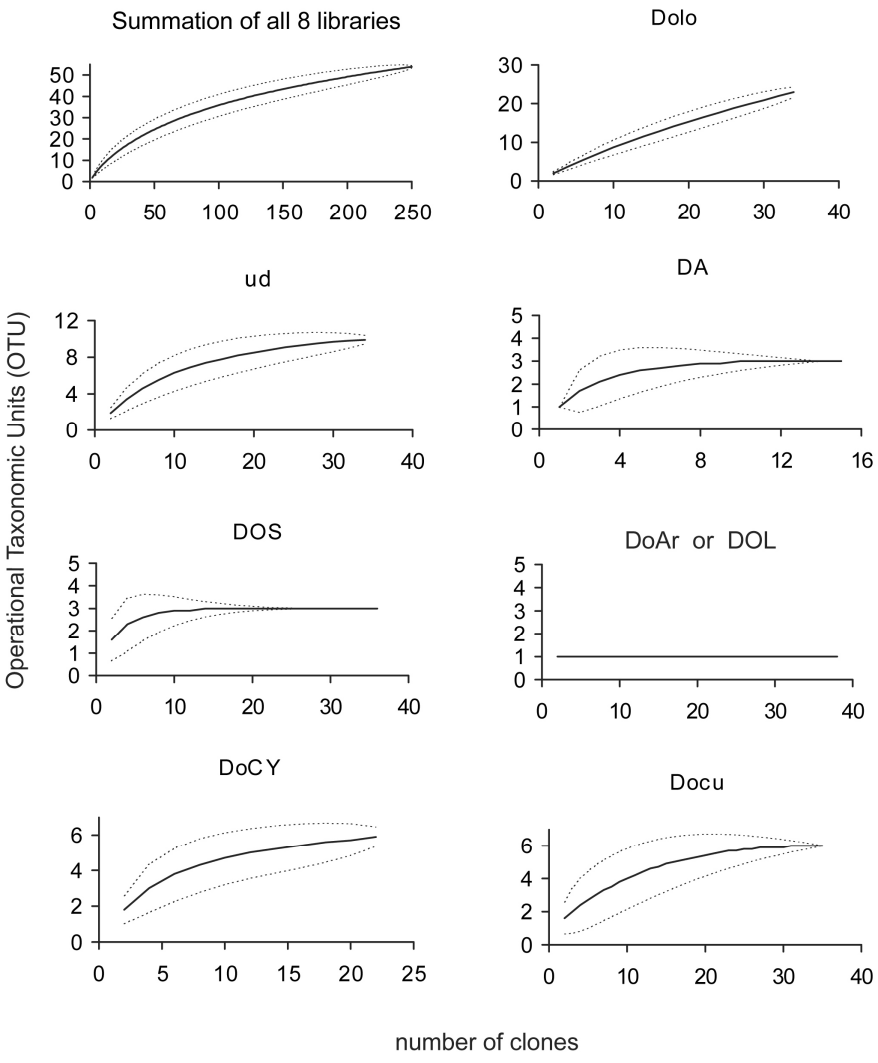

Figure 1 Rarefaction curves for the different libraries and for the sum of all clones obtained. The threshold is set at 3% sequence difference to distinguish between different OTUs. For clone library names, see Table 1.

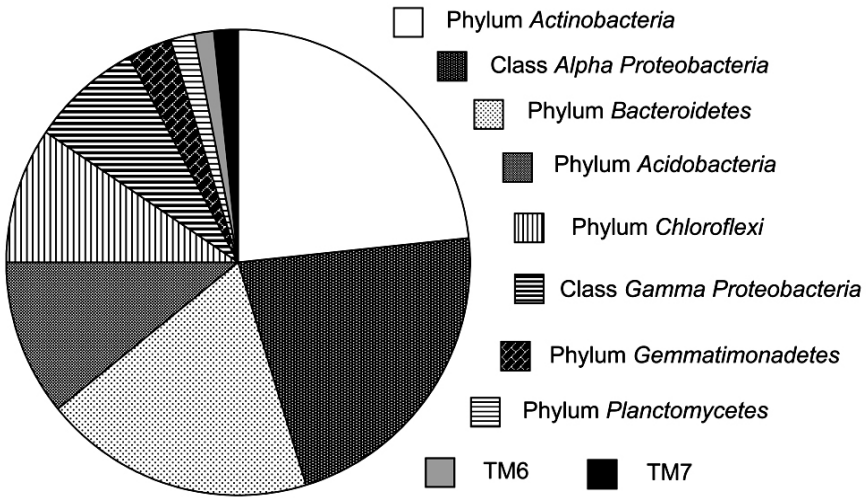

Figure 2 Distribution of phyla among the bacterial libraries "Dolo" and "ud". The five groups *Actinobacteria*, *Proteobacteria* (mainly *Alphaproteobacteria*), *Bacteroidetes*, *Acidobacteria*, and *Chloroflexi* are predominant in terms of the number of OTUs with 3% level distinction

ud10=7.8%, each percentage referring to the sum of non-oxigenic "ud" and "Dolo" clones), *Acidobacteria* (ud01= 9.4%), *Actinobacteria* (ud02=9.4%), and *Gamma Proteobacteria* (Dolo-06=7.8%; Table 2, and Fig. 2). None of these five most numerous sequences show a similarity to known SSU rRNA gene sequences of less than 95%. Several bacterial groups collectively account for a significant fraction of the total number of clones, while individual phylotypes are not particularly numerous. Nine phylotypes belong to the class *Alpha Proteobacteria* representing 22% of bacterial clones. Seven phylotypes belong to the phylum *Actinobacteria* and represent 23% of bacterial clones. Four phylotypes affiliate with uncultured *Chloroflexi*, accounting for 9.4% of the clones. Two phylotypes fall into the category of the phylum *Gemmatimonadetes* and consist of one clone each (3.1%). Phylotypes of *Planctomycetes*, of TM6 and of TM7 appear only once, each representing 1.6% of the bacterial clones. The phylogenetic position of the bacterial phylotypes is depicted in the trees in Fig. 3a and b.

Archaea

The archaeal library generated with the primer pairs 519f/1392r and 89Fb/915R (Table 1) resulted in three phylotypes—or four if ud14 and DOS_02 are counted as two separate phylotypes. They are 99.8% identical within their 420 bp fragment between positions 519 and 934 (Escherichia coli numbering). All the archaeal phylotypes found belong to the phylum *Crenarchaeota* and therein to the uncultured *Crenarchaeota* (Fig. 4). The phylotype of the clone DOS_02 amounts for the largest part of the crenarchaeal clones with 21 of 40 representatives (52.5%). It is followed by DOS_05 with ten clones (25%), DOS_21 with seven (17.5%), and ud14 with two (5%) out of 40 clones. All these clones show similarities of more than 98% with SSU rRNA gene sequences from the public

database, but for the time being, these are all uncultured archaeons. The closest named organism is *Cenarchaeum symbiosum*, an uncultured marine sponge symbiote [37], with similarities of 86% to ud14 and 81% to DOS_02, according to the EMBOSS Pairwise Alignment Tool provided by the EBI.

Eukaryotic Microorganisms

The primer combinations 8aF/1512uR and 8aF/1517r resulted in several eukaryotic sequences of SSU rRNA (Table 2). As the clones DA-04 and DA-15 are quite similar (97.2%), they are counted as one phylotype, likewise the clones DA-01 and DA-11, with 98.1% similarity. Hence, there are five different phylotypes, three of which belong to the class *Chlorophyta*, one to the order *Euamoebida* in the class *Lobosea*, and one to a moss in the division of the *Bryophyta* (Fig. 5). The phylotype of DoAr09 is 99.4% identical to *Saccamoeba limax* and the most numerous, with 39 out of a total of 83 eukaryotic clones (47%). Nevertheless, these numbers should not be overestimated, since they come from three combined clone libraries which were obtained under different conditions (chloroplasts not included). The moss represented by DOL_01 forms one third (33.7%) of the eukaryotic clones and is followed by the clones DA-01 (8.4%), DA-04 (7.2%), and DA-12 (3.6%), all belonging to the *Chlorophyta*. Two phylotypes, DA-01 and DA-12, have similarities of less than 95% to other sequences in public databases. DOL_01, DA-04, and DoAr-09 have NCBI-BLAST matches of more than 98%. Interestingly, DOL_01 (AB257668) is 99.2% identical to the *Hymenostylium recurvirostre* 18S rRNA (DQ629394), and Docu-30 (AB334295) is 99.7% identical to the *H. recurvirostre* chloroplast 16S rRNA (DQ629553), which suggests that protonemata of *Hymenostylium* prosper in the interstices of dolomite rock.

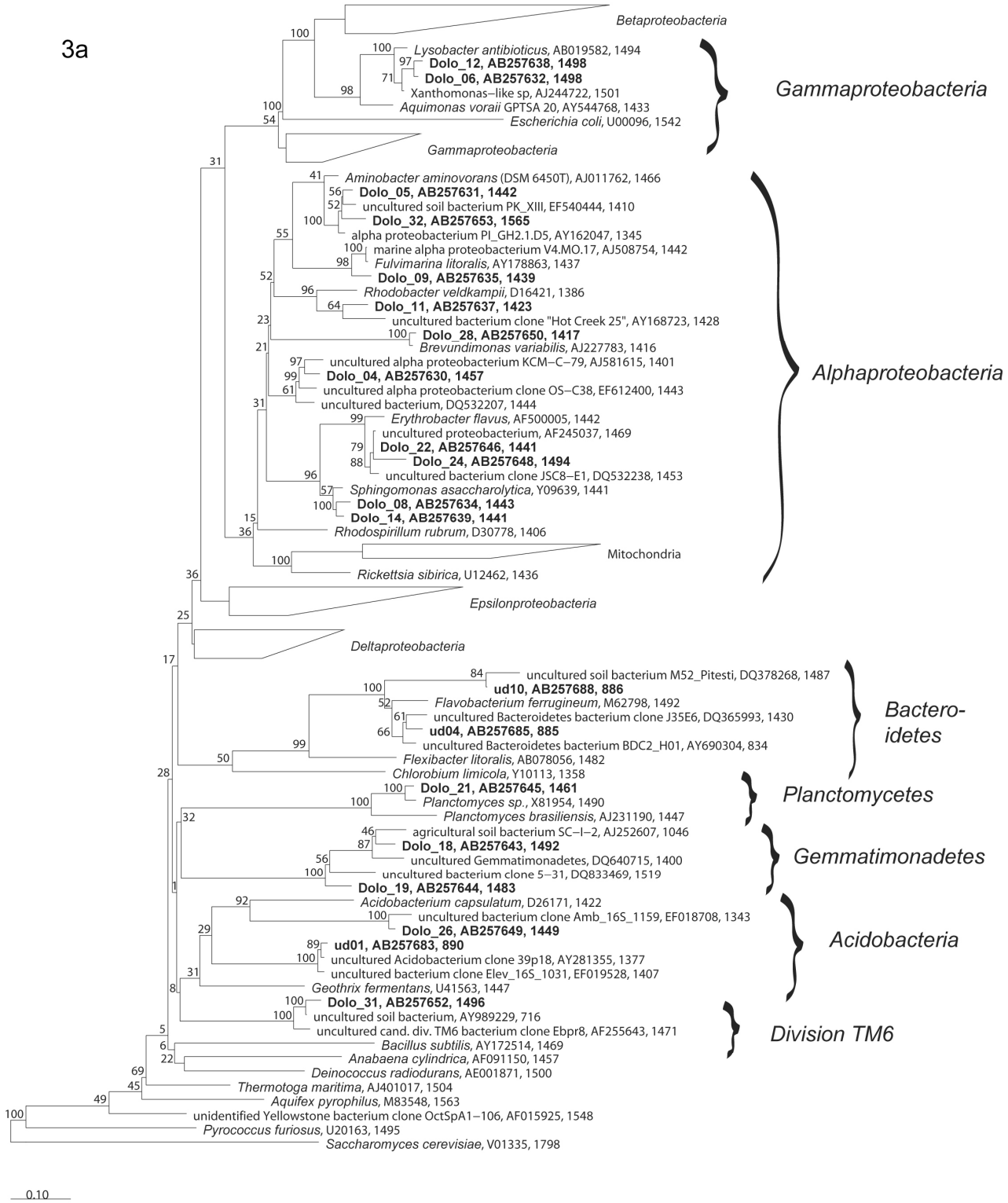

Figure 3 Phylogenetic tree with bacterial endolithic SSU rRNA gene sequences from alpine dolomite rock of the Piora Valley (in bold type) together with the closest relatives according to NCBI and ARB (tree calculated with ARB, Maximum Parsimony Method). The figures of Bootstrap values are given in percent. *Saccharomyces cerevisiae* is used to root the tree. Accession numbers and the length of the sequences (nucleotides) are indicated after the names. **a** part 1, **b** part 2

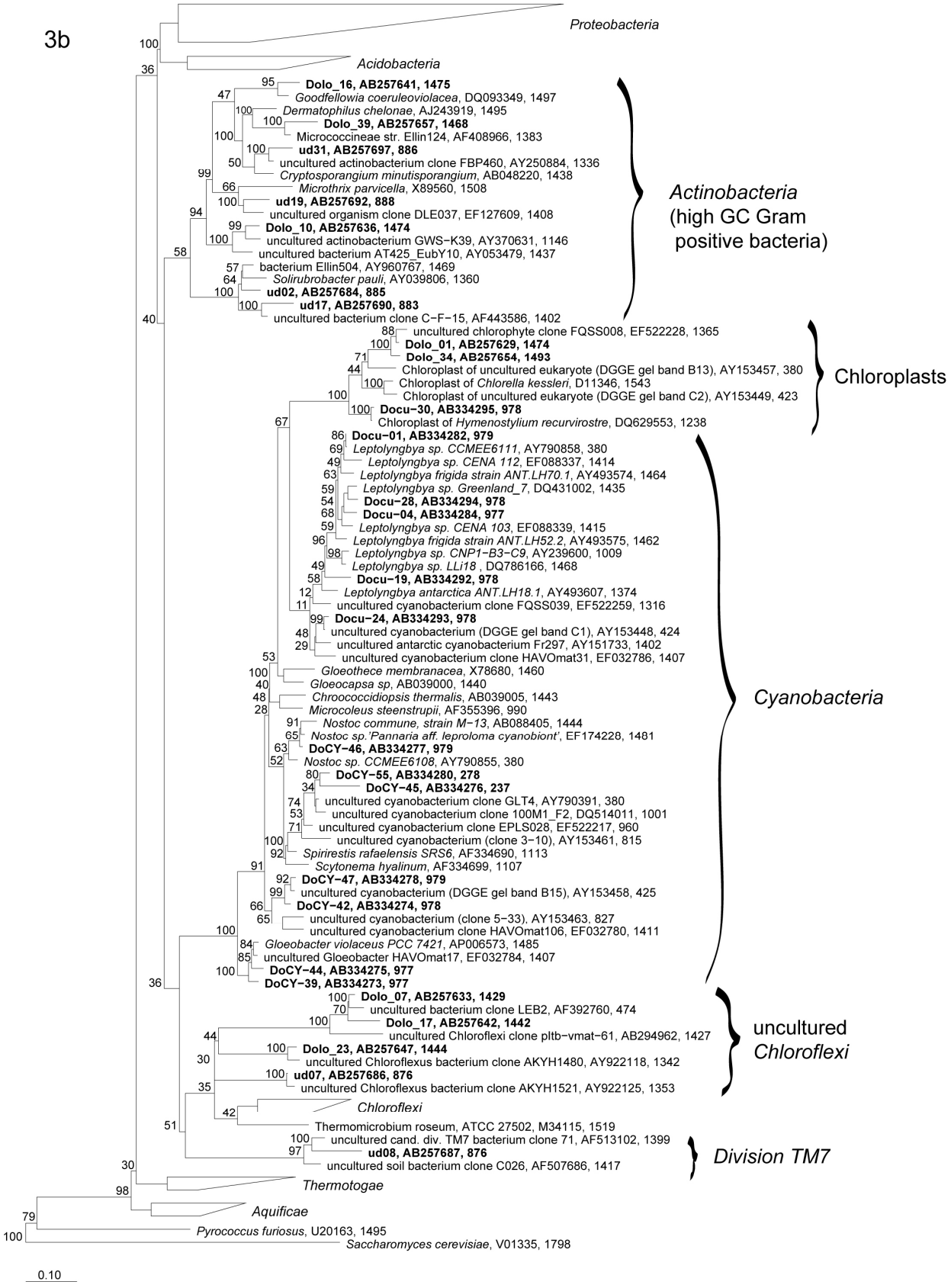

Figure 3 (continued)

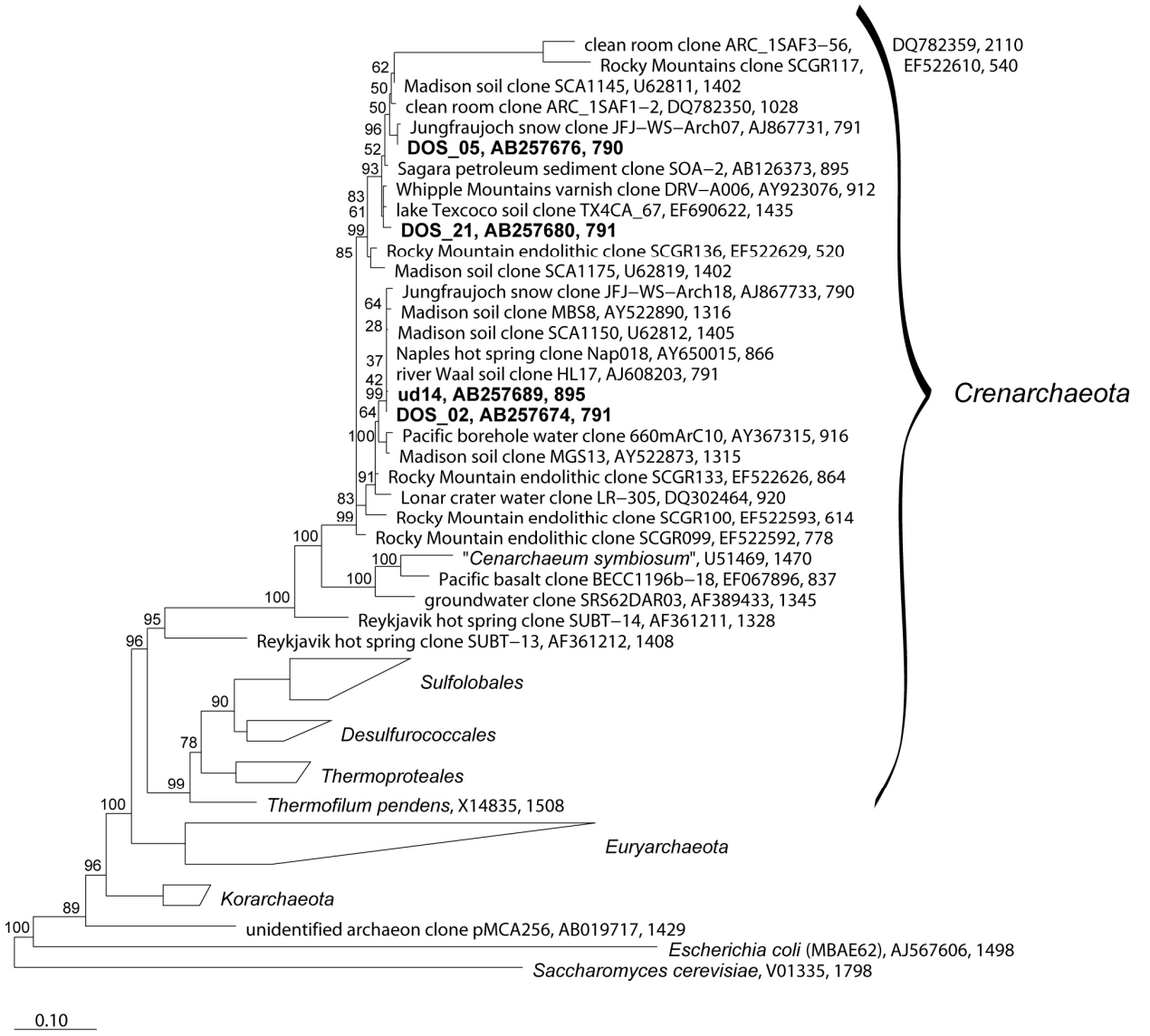

Figure 4 Phylogenetic tree of archaeal endolithic SSU rRNA gene sequences obtained from alpine dolomite rock of the Piora Valley (in bold type) together with other sequences of *Archaea* (tree calculated with ARB, Maximum Parsimony Method). All sequences found fall into the group of uncultured *Crenarchaeota*. *E. coli* and *S. cerevisiae* are used as the outgroup. The figures of Bootstrap values are given in percent. Accession numbers and the length of the sequences (nucleotides) are indicated after the names

Cyanobacterial Libraries

Two libraries were constructed with the specific primers CYA359F/CYA1342R. One came from a direct extraction of DNA from dolomite rock (DoCY) as described before, the other was obtained from an enrichment in a ten times diluted cyanobacterial BG11 medium seeded with rock material containing an endolithic band (Docu). 16S rRNA gene amplification, cloning, and sequencing yielded five different *Leptolyngbya* species (Docu-01, Docu-04, Docu- 19, Docu-24, Docu-28) as well as a chloroplast of the moss *Hymenostylium recurvirostre* (Docu-30, 99.7%). The DoCY

cloning yielded six different phylotypes related to *Nostoc* (DoCY- 46), *Gloeobacter* (DoCY-39 and DoCY-44), uncultured *Spirirestis* (DoCY-45 and DoCY-55), and an uncultured cyanobacterium (DoCY-42). The cyanobacterial sequences are included in the phylogenetic tree depicted in Fig. 3b.

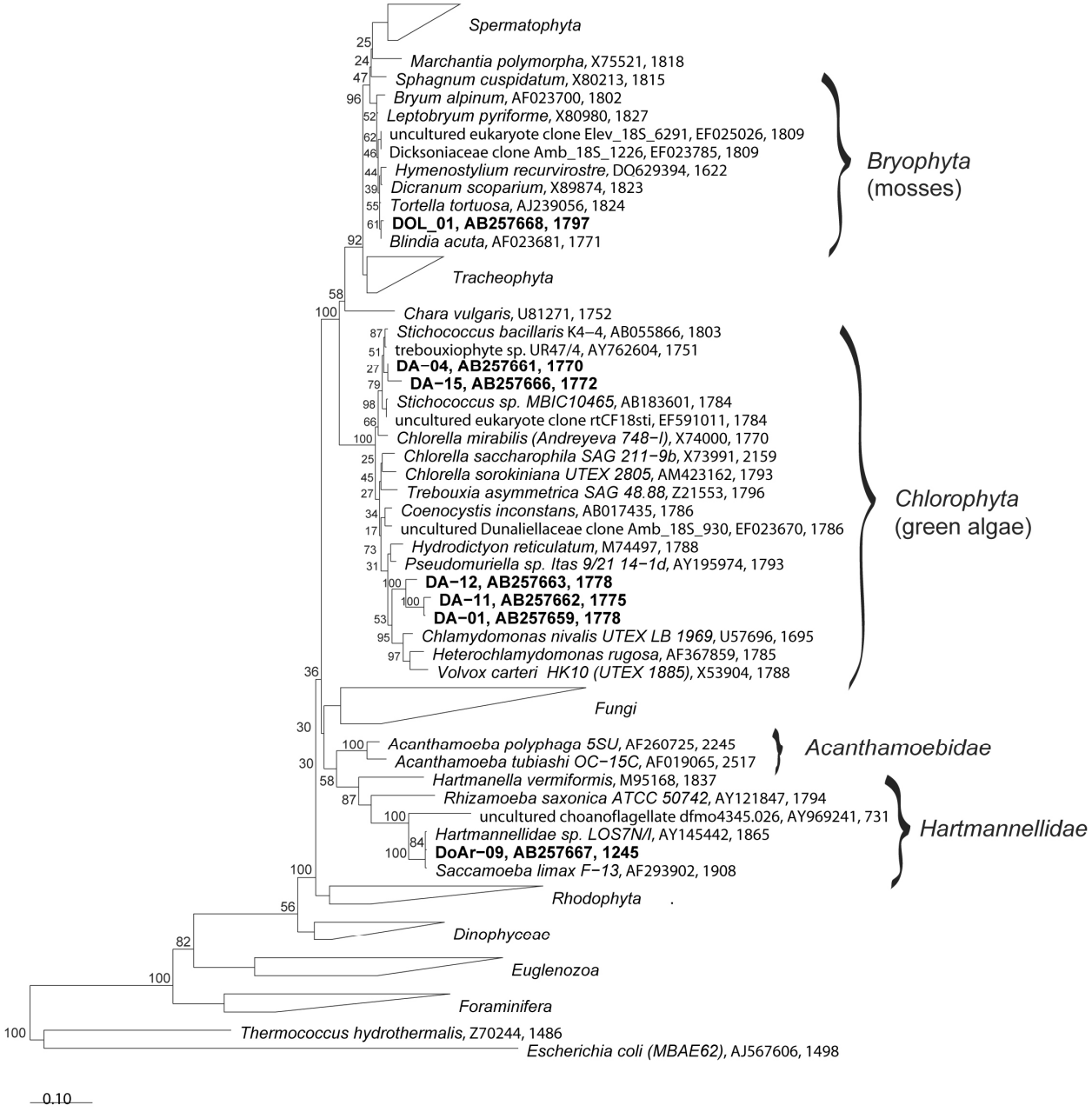

Figure 5 Phylogenetic tree of eukaryotic endolithic SSU rRNA gene sequences obtained from alpine dolomite rock of the Piora Valley (in bold type) together with other sequences of *Eukarya* (tree calculated with ARB, Maximum Parsimony Method). Accession numbers and the length of the sequences are indicated after the names. *E. coli* is used to root the tree. The figures of Bootstrap values are given in percent.

Discussion

Many endolithic ecosystems were studied in the past century, focusing mainly on algal and cyanobacterial diversity, by use of culture techniques and microscopic morphotypes for identification [96]. As the various stress factors present in endolithic sites may induce variations in size, color, and morphology, one cannot rely on morphological properties *in situ* or after cultivation. *Gloeocapsa sanguinea/alpina* changes its color from red (*G. sanguinea*) to blue (*G. alpina*), depending on the environmental pH level [45]. Morphological information alone may substantially mislead taxonomic identification [65]. Neither can pure culture techniques cover the full biodiversity, since in such a community culture, replication times of different species vary considerably, and mutualistic relations between species may get lost. Furthermore, it is questionable whether the better known epilithic microorganisms differ from the endolithic ones, which are thought to be restricted to the subsurface only. As it has, so far, hardly been possible to culture most environmental microorganisms, culture-independent molecular methods are suitable to obtain more information on the bacterial diversity. Walker and Pace suggest that, compared to other terrestrial ecosystems such as soil, endolithic communities in the Rocky Mountains, the Antarctica or the ones described here, are relatively simple systems with a rather restricted diversity. However, they also admit that molecular surveys do not completely sample the genetic diversity of a community [90].

Diels [19] and Jaag [45] found cyanobacteria in European Dolomite sites, Bell [7] in semi-arid regions and deserts in the southwest of the United States, Nienow and Friedmann [64] in the Antarctica, and Ferris and Lowson [22] as well as Gerrath et al. [31, 32] in limestone of the Niagara escarpment, all of which were classified by microscopy and culture techniques. Only a few of those genera have been confirmed with molecular methods. In endolithic habitats, cyanobacterial species related to *Plectonema* [17] and *Acaryochloris* [18] have been found as well as species related to *Anabaena*, *Chroococcidiopsis*, *Microcoleus*, *Nostoc*, and *Scytonema* [82]. The relationship between most of these sequences and the cultured strains is less than 96%. Up to now, Walker and Pace [89] have only found phylotypes "considerably different" from cultivated cyanobacteria. They have discovered two novel clades of specific endolithic cyanobacteria which are related to cultivated strains with less than about 94% sequence similarity {Owl Canyon Sandstone clone OCSS038 (EF522486) as compared with *Spirirestis rafaelensis* (AF334690)}. Lists of cultivated species and those of sequenced SSU rRNA genes hardly ever overlap, suggesting that species easy to cultivate may be the rare ones in nature. Norris and Castenholz [65] isolated endolithic phototrophs from rock material by culture techniques. Their list contains *Gloeocapsa*, very common in dolomite rock, as well as *Schizothrix*, *Nostoc*, and *Leptolyngbya*; all these genera were already mentioned by Jaag [45] or found with molecular methods by Sigler et al. [82]. However, about one third of the cultures listed by Norris and Castenholz have a similarity of less than 97% to the closest relatives known, and according to currently used criteria [84] may

be considered to be new species. This indicates that the bacterial diversity in most ecosystems must be larger than what has so far been detected by microscopy or cultivation as well as by sequencing.

By using specific cyanobacterial primers (CYA359F and CYA1342R), we found 11 phylotypes of cyanobacteria and three different sequences of chloroplasts of two green algae and one moss (Table 2). The cyanobacterial sequences indicated as closest cultivated relatives *Gloeobacter violaceus*, *Spirirestis rafaelensis*, several *Leptolyngbya* sp., *Nostoc edaphicum*, and *Nostoc commune*. *Microcoleus steenstrupii* was found to be related to the clones DoCY-45 and DoCY- 55, which were difficult to sequence and are only available as short sequences of about 200 bp. Sequences from the same sampling site, obtained earlier, suggest that *Microcoleus steenstrupii* as well as relatives of *Nostoc* PCC7120, of several *Chroococcidiopsis* sp., and of *Chlorella* sp. are also present there [82]. Sigler's DGGE band C1 obtained from an enrichment culture (AY153448) is now seen as the closest relative of our clone Docu-24. Both of them represent so far uncultivated cyanobacteria with 99.8% similarity between each other. The closest known cultivated strain to "band C1" is *Leptolyngbya* sp. PCC 9221 (94%), which confirms that there is still a gap in our knowledge as far as cultivated strains and collected environmental sequences are concerned. Sigler's sequence of band 15 (AY153458) now shows the closest similarity to clone DoCY-47 (AB334278) while bands 3 and 14 come closest to clone 46C-WNS (AB374402), which was gathered from a very similar environment in the Grisons, Switzerland. Interestingly, we also found a single chloroplast sequence, Docu-30 (AB334295), which corresponds 99.7% with a known chloroplast sequence of the moss *Hymenostylium recurvirostre* (DQ629553). This is affirmed by the presence of the 18S rRNA gene sequence of clone DOL_01 (AB257668) which is similar to the 18S rRNA gene sequence of *Blindia acuta* (AF023681) and of *H. recurvirostre* (DQ629394) by 99.5% and 99.2%, respectively.

Most environmental information on endolithic microorganisms is available on cyanobacteria. Clusters of *Leptolyngbya* are widely present in broad variations in all investigated ecosystems, in endolithic communities in the Rocky Mountains, in travertine of the Yellowstone National Park, in deep-sea basalt, and in alpine Piora dolomite [55, 65, 82, 89, and this paper]. *Nostoc* type filamentous organisms have been found in Piora and the Yellowstone, while relatives of coccoid *Gloeobacter* were observed in Piora and the Antarctica. *Gloeocapsa*, *Synechococcus*, *Synechocystis*, and *Chroococcidiopsis* are also present in all the above-mentioned systems but have not been detected in this study.

Little is known about the biodiversity of the heterotrophic bacterial communities accompanying the phototrophs. They were not dealt with in older studies for technical reasons. Sigler et al. [82] mentioned a large number of “non-cyanobacterial" clones without giving details. The phylogenetic tree (Fig. 3a and b) shows that in spite of the hostile environment, the heterotrophic endolithic population is quite diverse and consists of many different species. The cloning yielded 31 different chemotrophic bacterial clones with only a few doublets. This and the

rarefaction curves of the clone libraries "Dolo" and "ud" indicate that the inventory of new sequences is far from complete (Fig. 1). It contrasts with the organismic composition found in antarctic endolithic communities, where in communities with cyanobacteria as primary producers only two heterotrophic groups, the *α- proteobacteria* and the *Thermus-Deinococcus* group, were predominant besides the *Cyanobacteria*. The three groups together contributed to over 80% of the communities [17]. It remains to be tested whether it is possible to find more phylotypes in the McMurdo Dry Valleys or in the Piora dolomite by using different DNA extraction methods and different primers for the SSU rRNA gene. Using primer 1524r, for instance, instead of primer 1525r, with a difference of one base at the 3-prime end, already results in a strongly decreased number of detected cyanobacteria.

Most Piora sequences did not closely match with known sequences, and none of them were fully identical with a known sequence. The phylogenetic composition of the endolithic communities in Swiss dolomite was broader than the one in the Rocky mountains [89] with many phylotypes in the group of *Actinobacteria*, of *Alphaproteobacteria*, of *Bacteriodetes*, and of *Acidobacteria*. The group of *Actinobacteria* make up 23% of all phylotypes found in Piora dolomite, with a similar occurrence in the Rocky Mountains [89], on a wall in Fairy Cave, Glenwood Springs, CO, USA [3], and in rock varnish of the Whipple Mountains [48], but with 44%, they are more frequent in limestone of Ek Balam, Yucatan, Mexico [62] and with 65% predominant in rocks of the geothermal environment of the Yellowstone Park [88]. An explanation for the high fraction of *Actinobacteria* could be their strong cell wall and the capability of forming spores. Their high GC-content is also an advantage in extreme environments. In the dolomite of Central Switzerland, the overall sequence similarity of non-phototrophic prokaryotes was 94.9%; 40% of the bacterial clones and 45% of the chemotrophic ones showed a similarity of less than 95% to known SSU rRNA gene sequences. The highest similarity to cultured strains has been found in clones Dolo-40 and Dolo-28 with similarities of 99.4% and 98.8%, respectively, they are related to *Brevundimonas variabilis*, an α-proteobacterium. The lowest degree of similarity as compared with known 16S rRNA genes showed the clones Dolo-07, Dolo-17, and Dolo-29 with similarities of around 84%.

The observation of an *in vivo* absorption peak at about 720 nm in the pigments of the endolithic populations [42] suggests the presence of organisms from the branch of green nonsulfur phototrophs. These organisms were originally thought to live only in extreme environments such as hot springs [9, 38, 39, 70, 71], but some time ago, they were also found in temperate and even cold environments, such as wastewater treatment systems [4, 8, 80], the deep ocean [33], endolithic systems [17, 67, 89], as well as subsurface soil (paleosol) at a depth of 188 m [14]. Our sequence data confirm the presence of several green nonsulfur strains in the dolomite rock of the Piora Valley.

As in Antarctic endolithic communities [17, 83], except for *Cyanobacteria* and *Actinobacteria*, many phylotypes appeared in low numbers or even just as one, suggesting that the diversity must be substantially larger than presented by the clone libraries. This contrasts with

some of the rarefaction curves obtained (Fig. 1), which level off rapidly. We assume that this rapid flattening of some curves in Fig. 1 is due to technical limitations such as biased DNA extractions and/or insufficiently fitting amplification primers for the communities in question.

While Smith et al. [83], de la Torre et al. [17], and Sigler et al. [82] did not describe any *Archaea* in endolithic communities, *Crenarchaeota* phylotypes were found in the Rocky Mountains and in deep-sea basalt [55, 89]. In the phylogenetic tree with the archaeal branch (Fig. 4) the three sampling sites show a different distribution. Together with sequences from Australian marine stromatolites [67] and other uncultured *Crenarchaea*, samples from marine basalt {clone BECC1196b-18 (EF067896) as representative} group closely around *Cenarchaeum symbiosum*. On the other hand, the archaeal clones from the Rocky Mountains partially group around our clones ud14 and DOS_02 or form a slightly different group clustered around the clone "ARC_1-SAF3-56" (DQ782359) from a clean assembly room for NASA spacecraft [61] but are still closer to ud14 than to the basalt group. Interestingly, many other locations all over the world harbor *Crenarchaea* similar to the ones in the Piora dolomite, such as snow from Jungfraujoch in the Swiss Alps (AJ867733), for example, or soil from a rarely flooded plain by the river Waal in The Netherlands (AJ608203), or slit from a hot spring near Naples (Italy; AY650015), or the ODP 892 b borehole in the Pacific (AY367315), or soil in an agricultural research station in Madison (USA; U62812), or soil in the former Lake Texcoco close to Mexico City (EF690622), or in excaved material from a borehole, 200 m deep, of an oil drilling project in Japan (AB126373), or in the sediment of the Lonar Crater Lake in India (DQ302464).

On the whole, the archaeal sequences from the arid endolithic sites [present study and 89] are more related to each other than to endolithic organisms from aquatic sites [55]. A similar clustering has been observed in the group of the *Cyanobacteria*. The phylogenetic cluster formation of clones in similar habitats is more common than that of clones which live in different environments and are geographically further apart from each other. This indicates that both, geographical distances of the habitats and site-specific environmental factors have an influence on the biogeography of the organisms.

Among the heterotrophs, phagotrophic protists, mainly ciliates and flagellates, play an important role in the nutrient cycle as consumers of bacteria in aquatic environments. It has recently been discovered that *Amoeba* feed on cyanobacteria [97]. It is, thus, of special interest to find such consumers also in dry endolithic environments, where cyanobacteria form a large part of the biomass.

Conclusion

The results presented in this paper demonstrate that the bacterial diversity in endolithic habitats, especially of chemotrophic, nonpigmented organisms, is considerable but has been hidden and,

therefore, underestimated previously. As most of the sequences have only been found once or in low numbers, a much greater diversity than the one described here may be expected. The finding of some ribosomal sequences of the crenarchaeal branch demands for a more detailed study of the *Archaea*.

Acknowledgements

We are grateful to Steven M. Holland for providing his program Analytic Rarefaction as well as to John Marti for some revisions of the manuscript. And last but not least, we would like to thank the reviewers for their helpful comments and corrections.

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4. Discussion

4.1. Rock-inhabiting microorganisms

Discussions on subsurface microorganisms started in the 1980ies with the search for safe depositories of radioactive waste (Barraclough et al., 1976; U.S. Department of Energy, 1983; U.S. Department of Energy, 1986; Chapelle et al., 1987; Colwell, 1989; Bachofen, 1996; Krumholz et al., 1997; Bachofen et al., 1998; Stroes-Gascoyne & Sargent, 1998). The finding of microbial life far below the surface and in the deep subsurface was a surprise for many microbiologists as the observations of Lipman were not widely known (Lipman, 1928). Nobody had expected such a diverse microbial life inside of rocks and stones. Most stones, even hard rocks like granite, are porous, and have cracks and fissures, providing space for colonization by microorganisms. This is especially true for the weathered dolomite in the Piora Valley discussed in this thesis. In contrast to deep subsurface microorganisms, which live chemotroph on energy-rich substrates from the interior of the earth, a photosynthesis-driven microbial ecosystem develops within the uppermost cm of the rock. The crystalline grains of the dolomite permit transfer and scattering of sunlight deeper than 10 mm below the surface. Water and air penetrate as well. The rock surrounding the organisms in the micro-cavities buffers large differences of temperature and protects from strong solar radiation. It provides a niche of habitat that is less harsh as its surrounding. The endolithic environment, as described in the dolomite of the Piora Valley and in similar environments in the Arctic, Antarctic and high mountain regions are counted as extreme environments. Not astonishing they are assumed to be the closest analogs of habitats for life on other planets or moons in the universe like Mars, the moon Europe of Jupiter, the moons Titan and Enceladus of Saturn, the extrasolar planet Corot-7b, or the extrasolar "super-Earth" GJ1214b (Daily News & Analysis, December 17, 2009). Therefore, one of the first places to look for extraterrestrial life in the outer space would be the typical endolithic habitat (Parnell et al., 2002; Parnell et al., 2003; Parnell & Baron, 2004; Schulze-Makuch et al., 2008; Horneck et al., 2010)..

4.1.1. Endolithic microorganisms colonize rock surfaces that are exposed

On Earth, in recent years rock-inhabiting microorganisms have been found to be widespread. They colonize rock surfaces and the layers below in a wide variety of climates and environments. These range from hot deserts to Antarctica, from Alpine to submerged marine and freshwater rocks, and from caves to the surfaces of buildings and monuments. In general such lithobiontic microorganisms colonize rock surfaces that are exposed to the

atmosphere, not covered by soil or higher organisms such as lichens or mosses (Friedmann and Ocampo-Friedmann, 1984). This also holds true for the dolomite rocks in the Piora Valley where endolithic growth is found. Endolithic organisms are found in rocks irrespective of the slope and geographical orientation of its surface. The typical green-grayish bands a few millimeters below the surface are present all over. "Wherever you look you will find a sub-aerial biofilm" (Gorbushina and Broughton, 2009)

4.1.2. The endolithic habitat is a microcosm with a high diversity

Endolithic habitats often are insulated and form a protected niche within an extreme and inhospitable macro-environment as closed system and integral micro-cosmos upon which external conditions have only limited influence (Friedmann and Ocampo-Friedmann, 1984). Further they are regarded as simple model ecosystems that lack secondary consumers and predators (Friedmann and Ocampo-Friedmann, 1984). Walker and coworkers (2005, 2007a, 2007b) correlate this simplicity with a small diversity, which may not hold for every endolithic habitat. Our finding of an 18S rRNA gene sequence of an amoeba in the dolomite of the Piora Valley also indicates that predators are indeed present (Horath and Bachofen, 2009; Pointing et al., 2009). The question whether the diversity in an endolithic habitat is poor or rich is not easy to answer. At present time the complete microbial range has probably not been fully sampled. "The large population size and the rapid growth of prokaryotes provide an enormous capacity for genetic diversity." (Whitman et al., 1998). Heterogeneity is a feature of extreme environments which encourages diversity, particularly amongst the lower life forms that dominate extreme environments – food chains are short but these are not simple ecosystems (Evans, 2010).

4.1.3. Methodological and technical considerations; what is necessary to fully describe the diversity of a habitat?

In our point of view, in a molecular SSU rRNA sequencing approach, several parameters should be considered before acknowledging a full scan of the diversity:

- complete extraction of the DNA and prevention of its lysis during preparation
- use of a 100% universal primer pair
- non biased PCR
- efficiency of the cloning
- sequencing of all clones obtained

There are certainly even more parameters, also depending on the methods used, and at least the first two points are in general hardly achieved. Most of the time these goals are approached only asymptotically. Concerning "universal primers", a recent approach used very small primers, bacterial "mini-primers" for soil and microbial mat samples. Novel 16S rRNA gene sequences were found that would not have been detected with standard primers. Deeply divergent sequences were discovered with high frequency which included even representatives that define two new division-level taxa (Isenbarger et al., 2008).

4.1.4. How to select primer pairs to investigate environmental microbial communities?

In our project, adding a Guanosine at the three prime end of primer 1525r (Lane, 1991) transformed it into an oligomer that no longer aligned to most of the cyanobacterial DNA sequences of the SSU rRNA gene, making it no longer being "universal" (which can be doubted for any so called universal primer), but at the same time giving the advantage that the sequences of heterotrophic bacteria became better amplified. To test this proposal, one should use the established primer 1525r and the new primer 1524r in parallel and compare the two clone libraries. Kwok et al. (1990) showed that the 3'-terminal position in the primer is essential for controlling priming. Aligning the 16S rRNA gene sequences and searching for conserved regions, a majority of the conserved bases are not adjacent to each other (Baker et al., 2003). The longest string of totally conserved bases were found from position 788 to 797 (*E. coli* numbering after Brosius et al., 1978; 1981; sequence 5'-TTAGATACCC-3'), while in most areas of the gene absolutely conserved bases are present in strings of less than four nucleotides. Thus, no primer of sufficient length can be designed that will 100% match to all bacterial and/or all archaeal 16S rRNA gene sequences, meaning that none of the primers in current use are truly "universal" and no set of primers will be guaranteed to fully amplify all prokaryotic SSU rRNA genes (Baker et al., 2003). It is recommended to either concentrate only on single groups of microorganisms or to use several primer pairs and construct several clone libraries.

4.2. *Archaea* in endolithic habitats

Archaea are common inhabitants in many microbial ecosystems, however, they have not been described for endolithic habitats for a long time. Although archaeal primers have been described in the literature (Table 1) the design of new primers could lead to new unknown archaeal rRNA gene sequences. Forward and reverse primers starting near the beginning (5 prime end; 27f, 8aF, 89Fb) and the end (3 prime end; 1392r, 1512uR, 1517r,

1524r) of the SSU rRNA gene should lead to a complete as possible sequencing coverage. Primers at or close to the beginning and end of the SSU rRNA gene usually yield longer sequences that are more accurate to build phylogenies on (Ludwig W, Amann R, personal communication). While shorter partial sequences of SSU rRNA genes can be misleading, since often the shortest sequences result as best hits in a BLAST search (Altschul et al., 1997), although they often would not fit best, if their gene was sequenced completely. The close relationship between endolithic sequences from the Antarctica (Pointing et al., 2009) and the 400 bp long sequences from Piora dolomite (Sigler et al., 2003) raises the question whether only short sequences from the Piora Valley fit to the sequences of Pointing et al. while longer ones do not? Of course, the described observation is possible also among bacterial phylogeny, not only while investigating the archaeal SSU rRNA gene.

4.2.1. Primers for amplification of the archaeal 16S rRNA gene

The following remarks hold for *Bacteria* as well as for *Archaea*, they are discussed here for *Archaea*, as this phylum has been found only recently among endolithic organisms. Not all PCR primers at or close to the end of the archaeal or bacterial 16S rRNA gene may function: The annealing temperature should fit, the primer sequence should correspond to the target sequences, the primers should not be self-annealing, and ideally the primers of a reaction pair have the same annealing temperature. In table 1, published primers suitable for the amplification of archaeal 16S rRNA genes are compiled, concentrating on primers that anneal close to the start or the end of the ribosomal gene.

In general the primers listed are different, often only slightly. But still, even a slight difference, often only a single nucleotide and especially at the 3' end of a primer, can already select for remarkably different sequences as we have seen for the primers 1525r and 1524r.

Table 1. Primers that can be used to amplify archaeal SSU rRNA gene sequences

Specificy	Sequence (5'->3')	*E.coli* # Positions	Reference	Name	Anneal. Temp [*3)]
Archaea	5'-ATTCCGGTTGATCCTGC-3'	0006-22	Arahal et al. (1996)	D30	47℃
Archaea	5'-ATTCYGKTTGATCCYGSC-3'	0006-23	Petroni et al. (2000) / Ludwig	Arc6-23	46-53℃
Archaea	5'-ATTCCGGTTGATCCTGCCGG-3'	0006-25	Ronimus et al. (1997)	Arc25f	55℃
Archaea / Eukarya	5'-AACCTGGTTGATCCTGCCAGT-3'	0006-26	Medlin et al. (1988) *2)	PRIMER A	56-58℃
Archaea	5'- CTCCGGTTGATCCTGCC-3'	0007-23	Bruggraf et al. (1991)	Arc23f	52℃
"Nanoarchaea"	5'- CTCCCGTTGATCCTGCG-3'	0007-23	Hohn et al. (2002)	7mcF	52℃
Crenarchaea	5'- TCCCGGTTGATCCTGCCRG-3'	0007-25	Hershberger et al. (1996)	4Fa	55-58℃
Archaea	5'- TTCCGGTGGATCCYGCCRG-3'	0007-25	Massana et al. (1997)	20f	58-60℃
Archaea	5'- TTCCGGTTGATCCYGCCGGA-3'	0007-26	De Long (1992)	Arch21F	55℃
Archaea	5'- TTCCGGTTGATCCYGCCGGA-3'	0007-26	Giovannoni et al. (1988)	“archaebact probe”	56-58℃
Archaea	5'-GCGGATCCGCGGCCGCTGCAGA- -YCTGGTYGATYCTGCC-3'	0008-23	Barns et al. (1994)	23FPL	55℃
Archaea	5'- TCYGGTTGATCCTGCC-3'	0008-23	Eder et al. (1999) Huber et al. (2000)	8aF	60℃
Archaea	5'- TCYGGTTGATCCTSCC-3'	0008-23	Horath & Bachofen (2009)	8aF	60℃
Archaea	5'- TCCGGTTGATCCTGCC-3'	0008-23	Edgcomb et al. (2002)	A8F	49℃
Archaea	5'- TCCGGTTGATCCTGCC-3'	0008-23	Huber et al. (2006)	8Fa	49℃
Archaea	5'- TCCGGTTGATCCTGCCAG-3'	0008-25	Achenbach & Woese (1995)	(0008 Reverse)	53℃
Archaea	5'- TCCGGTTGATCCTGCCGG-3'	0008-25	Kolganova et al. (2002)	A8F	55℃
Archaea	5'- TCCGGTTGATCCTGCCGG-3'	0008-25	McInnery et al. (1995)	AB	55℃
Archaea	5'- TCYGKTTGATCCYGSCRGAG-3'	0008-27	Embley et al. 1992	1Af	52-60℃
Archaea	5'- CTGGTTGATCCTGCCAG-3'	0009-25	Achenbach & Woese (1995)	0025e Forward	55℃
Archaea	5'- CTGGTTGATCCTGCCAG-3'	0009-25	Vetriani et al. (2003)	16F	55℃
Archaea	5'- GTTTGATCCTGGCTCAGG-3'	0011-28	Achenbach & Woese (1995)	(0011 Reverse)	50℃
Archaea	5'- TCCGGCRGGATCAACCGGAA-3'	0026-7	Amann et al. (1995)	4f	53.6℃
Crenarchaea	5'-ACGGCTCAGTAACRC-3'	0089	Buckley et al. (1996)	89Fb	48℃
Crenarchaea	5'- GGCTCAGTAACGCGTAGTC-3'	0089	Hershberger et al. (1996)	89F	53℃
Archaea	5'-ACKGCTCAGTAACACGT-3'	0109-125	Whitehead & Cotta (1999)	A109f	45-47℃
Archaea	5'- GCTSAGTAACACGTGG-3'	0112-128	Achenbach & Woese (1995)	0112a Forward	55°C
marine *Euryacheota*	5'-GAGATGGATTCTGAGACACGAA-3'	0333-	Suzuki et al. (2000)	ARCHGII-333F	59℃
Archaea	5'-TCCAGGCCCTACGGG-3'	0333-348	Achenbach & Woese (1995)	0348a Forward	50℃
marine *Crenarcheota*	5'- AGATGGGTACTGAGACACGGAC-3'	0334-	Suzuki et al. (2000)	ARCHGI-334F	59℃
Archaea	5'-GGCCCTACGGGGSGCASCAGGCGC-3'	0338-361	Giovannoni et al. (1990)	{338-361} A361f	88℃ / 71
Most *Archaea*	5'-ACGGGGYGCAGCAGGCGCGA-3'	0344-363	Casamayor et al. (2000)	ARC344F-GC	61℃
Archaea	5'-ACGGGGYGCAGCAGGCGCGA-3'	0344-363	Raskin et al. (1994) *[1)]	ARC344/ Arc363f	54℃
Archaea	5'- CGGGGYGCASCAGGCGCGAA-3'	0345-364	Burggraf et al. (1997)	344aF	60℃

Archaea	5'- CTGSTGCRCCCCGTAGGGCC-3'	0357-338	Burggraf et al. (1997)	338aR	62-64℃
Archaea	5'-TCGCGCCTGCGCCCCGT-3'	0360-344	Arahal et al. (1996)	D33	62-64℃
"universal"	5'-GTGCCAGCMGCCGCGG-3'	0515-530	Lane (1991)	530f	56-59℃
"universal"	5'-GTGYCAGCMGCCGCGGTA-3'	0515-532	Lane (1991) (modified)	"532f"	55-59℃
"universal"	5'-GTGYCAGCMGCCGCGCGGTAA-3'	0515-535	Takai & Sako (1999)	Uni515F	55℃
"universal"	5'- TGBCAGCMGCCGCGGTAA-3'	0516-533	Burggraf et al. (1997)	533F	53-57℃
"universal"	5'- CAGCAGCCGCGGTAATAC-3'	0519-536	Edwards et al. (1989)	536f ("pD")	53℃
"universal"	5'.gcttCAGCMGCCGCGGTAATWC-3'	0519-536	Fuhrman et al. (1992)	Uni 536f	66-67℃
Prokaryotes	5'-GTATTACCGCGGCTGCTG-3'	0536-519	Edwards et al. (1989)	519r ("pDr")	53℃
Prokaryotes	5'-GWATTACCGCGGCKGCTG-3'	0536-519	Lane et al. (1985)	519r ("A")	53-55℃
Archaea	5'-GSNGCYGGTRTTACCGCGGC-3'	0543-524	Burggraf et al. (1997)	524aR	58-64℃
marine *Euryacheota*	5'- TTAGGCCCAATAAAAKCGAC-3'	0573-554	Suzuki et al. (2000)	ARCHGII-554R	59℃
marine *Crenarcheota*	5'-CTGTAGGCCCAATAATCATCCT-3'	0575-554	Suzuki et al. (2000)	ARCHGI-554R	59℃
"universal"	5'-AAACTYAAAKGAATTGRCGG-3'	0907-926	Lane (1991) (modified)	926f	44-50℃
Archaea	5'-AAAGGAATTGGCGGGGGAGCAC-3'	0913-934	Casamayor et al. (2002)	915f	55℃
Archaea	5'- AGGAATTGGCGGGGGAGCAC-3'	0915-934	Stahl and Amann (1991)	Arch915	56℃
"universal"	5'-CCGYCAATTCCTTTRAGTTT-3'	0926-907	Lane (1991+1985) (modified)	907r	46-50℃
"universal"	5'-CCGTCAATTCMTTTRAGTTT-3'	0926-907	Lane et al. (1985)	907r ("B")	44-48℃
Crenarcheota	5'-CCCGCCAATTCCTTTAAGTTTC-3'	0927-906	Jurgens et al. (1997)	ARC906R	53℃
Archaea	5'-GTGCTCCCCCGCCAATTCCT-3'	0934-915	Casamayor et al. (2002)	915r = Arch915	61°C
Archaea	5'-GTGCTCCCCCGCCAATTCCT-3'	0934-915	Stahl and Amann (1991)	ARC915R	56℃
Archaea	5'-YCMGGCGTTGAVTCCAATT-3'	0976-958	based on Lane (1991) unpublished	958r	57℃
Archaea	5'-YCCGGCGTTGAMTCCAATT-3'	0976-958	De Long (1992)	Arch958R	55℃
Archaea	5'-GGYRSGGGTCTCGCTCGTT-3'	1119-1101	Burggraf et al. (1997)	1119aR	55-60℃
Archaea	5'-CGGTGAATACGTCCCTGC-3'	1369-1386	Suzuki et al. (2000)	ARCH1-1369F	59℃ / 53
Archaea	5'-CGGTGAATATGCCCCTGC-3'	1369-1386	Suzuki et al. (2000)	ARCH2-1369F	59℃
Prokaryotes	5'-CTTGTACACACCGCCCGTC-3'	1389-1407	Suzuki et al. (2000)	TM-1389F	55-58℃
Prokaryotes	5'-CTTGYACACACCGCCCGTC-3'	1389-1407	unpuplished	Prok1389s	63℃
"universal"	5'-TGYACACACCTCCCGT-3'	1391-1406	Lane (1991)	1406f	46-49℃
"universal"	5'-TGYACACACCGCCCGT-3'	1391-1406	Lane (1991) modified	1406f	49-51℃
"universal"	5'- CGGTGTGTGCAAGGRGC-3'	1401-1385	Munson et al (1997)	1404R	63-53℃
"universal"	5'- ACGGCGGTGTGTRC-3'	1405-1392	Lee et al. (1996)	1392r ("1406RU")	55℃
"universal"	5'- ACGGGCGGTGWGTRCAA-3'	1406-1390	Baker et al. (2003)	UA1406R	49-52℃
"universal"	5'- ACGGGCGGTGWGTRCAA-3'	1406-1390	based on Lane (1991)	1390r	49-52℃
"universal"	5'- ACGGGCGGTGTGTRCAA-3'	1406-1390	Huber et al. (2000)	1406uR	49-52℃
"universal"	5'- ACGGGCGGTGTGTAC-3'	1406-1392	Edwards et al. (1989)	1392r ("pGr")	47℃
Prokaryotes	5'-…agaACGGGCGGTGTGTRC-3'	1406-1392	Fuhrman et al. (1992)	Proc 1392r	64-66℃
"universal"	5'- ACGGGCGGTGTGTRC-3'	1406-1392	Lane et al. (1985)	1392r ("C")	47-50℃
"universal"	5'- GACGGGCGGTGTGTRCAA-3'	1407-1390	based onLane (1991)	1390r	53-55℃

"universal"	5'- GACGGGCGGTGTGTRCA-3'	1407-1391	Reysenbach & Pace (1995)	(1407-1391)	52-54℃
"universal"	5'- GACGGGCGGTGTGTRC-3'	1407-1392	Wilmotte et al. (1993)	1392r ("15")	55℃
"universal"	5'- TGACGGGCGGTGTGTRCAA-3'	1408-1390	Takai & Sako (1999)	Uni1408R	55℃
Prokaryotes	5'- TACCTTGTTACGACTT-3'	1507-1492	based on Lane (1991)	Prok1492as	38℃
Prokaryotes	5'- GHTACCTTGTTACGACTT-3'	1509-1492	Ronimus et al. (1997)	Prok1492r	55℃
Prokaryotes	5' -GYTACCTTGTTACGACTT-3'	1509-1492	Arahal et al. (1996)	D56	55℃
Prokaryotes	5'- GGCTACCTTGTTACGACTT-3'	1510-1492	Edgcomb et al. (2002)	A1492R	49℃
Prokaryotes	5'- GGYTACCTTGTTACGACTT-3'	1510-1492	Huber et al. (2006)	1492Ra	51-53℃
Prokaryotes	5'- GGTTACCTTGTTACGACTT-3'	1510-1492	Reysenbach & Pace (1995) / Achenbach & Woese (1995)	1492 Reverse	51-53℃
"Nanoarchaea"	5'- CGGCTACCTTGTGTCGACTTAG-3'	1511-1490	Hohn et al. (2002)	1511mcR	57℃
Prokaryotes	5'- ASRGYTACCTTGTTACGACTT-3'	1512-1492	based on Lane (1991)	Prok1492r	49-52℃
"universal"	5'- ACGGHTACCTTGTTACGACTT-3'	1512-1492	Eder et al. (1999) Huber et al. (2000)	1512uR	60℃
Prokaryotes	5'- ACGGNWACCTTGTTACGAGTT-3'	1512-1492	McInnery et al. (1995)	1492r ("UN")	55℃
"universal"	5'- ACGGHTACCTTGTTACGACTT-3'	1512-1492	Rudolph et al. (2001)	1512uR	50-52℃
"universal"	5'- ACGGCTACCTTGTTACGACTT-3'	1512-1492	Vetriani et al. (2003)	"1517R"	55℃
"universal"	5'- ACGGHTACCTTGTTACGACTT-3'	1512-1492	Weisburg et al. (1991)	rP1,rP2,rP3	42℃
Prokaryotes	5'-TACRGYTACCTTGTTACGACTT-3'	1513-1492	based on Lane (1991)	1492r v2	49-53℃
Prokaryotes	5'-TACGGYTACCTTGTTACGACTT-3'	1513-1492	Lane (1991)	1492r	51-53℃
Archaea / some Eukarya	5'- ATCCAGCCGCAGRTTC-3'	1532-1517	Horath & Bachofen (2009)	1517r	50℃
Archaea	5'- GAGGTGATCCAGCCGCAGRTT-3'	1538-1518	based on Lane (1991)	Arch1518r	56-58℃
Archaea	5'- GAGGTGATCCTGCCGCAG-3'	1538-1521	based on Lane (1991)	Arch1521r	55℃
Archaea	5'- GGAGGTGATCCAGCCGCAG-3'	1539-1521	Petroni et al. (2000) / Ludwig	"Arc1521r"	58℃
Archaea	5'- GGAGGTGATCCAGCCG-3'	1539-1524	Bruggraf et al. (1991)	"Arc1524r"	51℃
Bacteria and Archaea	5'-AAGGAGGTGATCCANCCNCACC-3'	1541-1520	Giovannoni et al.(1990) / DeLong (1994)	1520r ("OX2")	57-60℃
Prokaryotes	5'-AAGGAGGTGATCCAGCCGCA-3'	1541-1522	Edwards et al. (1989)	1522r ("pHr")	56℃
Prokaryotes	5'-AAGGAGGTGATCCRGCCGCA-3'	1541-1522	Suzuki et al. (2000)	PROK-1541R	59℃
Prokaryotes?	5'-AAGGAGGTGATCCAGCCGCA-3'	1541-1522	Wilmotte et al. (1993)	1522r ("16")	55℃
Prokaryotes	5'-AAGGAGGTGATCCAGCC-3'	1541-1525	Achenbach & Woese (1995)	1525r	47-49℃
Prokaryotes	5'-AAGGAGGTGWTCCARCC-3'	1541-1525	Lane (1991)	1525r	47-49℃

*1) Raskin et al. (1994) used in their oligonucleotide probe also an inosine (I) which can be put equal to using an adenosine or a guanine at that position, meaning a purine (R). The forward primer sequence (“Arc363f”) is then the Reverse Complement Strand of the probe ARC344.
*2) used for *Archaea* by DeLong et al. (1994)
*3) In case no annealing temperature is given by the reference (black figures), the basic melting temperature (Tm) has been calculated with the formulas given on: "http://www.basic.northwestern.edu/biotools/oligocalc.html" (pink figures). For sequences less than 14 nucleotides long, **Tm= (wA+xT) * 2 + (yG+zC) * 4** where w,x,y,z are the number of the bases A,T,G,C in the sequence, respectively (Marmur and Doty, 1962). For sequences longer than 13 nucleotides, **Tm= 64.9 +41*(yG+zC-16.4)/(wA+xT+yG+zC)** (Wallace et al., 1979; Sambrook & Russell, 2001). Both equations assume that the annealing occurs under standard conditions of 50 nM primer concentration, 50 mM Na^+ concentration, and pH 7.0.

4.2.2. Different primer sets lead to different results

When primer sets in eight studies covering different environments are compared, the archaeal diversity in non-extreme habitats (moderate temperature, salinity, and pH) measured by sequencing 16S rRNA genes is generally restricted to a few, often only one or two phylogenetic clusters per environment (Crump and Baross 2000). Most of these uncultivated Archaea tend to form groups that are specific to each habitat type (Buckley et al., 1998), such as soil (Jurgens et al., 1997; Bintrim et al., 1997), freshwater and marine sediment (MacGregor et al., 1997; Munson et al., 1997; Vetriani et al., 1998), and marine plankton (DeLong, 1992; Fuhrman et al., 1992). In all of these studies different primer pairs were used, therefore the different results may be not only due to the different environments but to different primer sets, too (Table 2).

Table 2. Primer pairs used by different laboratories to search for archaeal 16S rRNA gene sequences, with names of the clusters obtained and their environment

Primer pair used	specific cluster fetched	environment	clones	Reference
Arch21F / Arch958R	SBAR, WHAR, widespread occurrence of "unusual *Archaea*" (*Crenarchaeota* and *Euryarchaeota*)	marine oxygenated coastal surface waters of North America (Woods Hole and Santa Barbara Channel)	100 analyzed, 6 sequenced	DeLong (1992)
Uni536f / Proc1392r	NH49 (500m depth) NH25 (100m depth), marine plankton, *Crenarchaeota* (Pyrodictium) and *Euryarchaeota* (Haloferax)	East Pacific, 650 km west of San Diego at 500m and 100m depth (31°50'N 124°6'W)	37 analyzed	Fuhrman et al. (1992)
Arch21F / Arch958R	ANTARCTIC	Marine surface waters of Antarctica	14 analyzed	DeLong et al. (1994)
23FPL / 1492r	SCA; nonthermophilic *Crenarchaeota*	Agricultural soil, Wisconsin	35 analyzed	Bintrim et al. (1997)
Arch21F / Arc906R	FFSB; nonthermophilic *Crenarchaeota*	Forest soil, Finland	9 sequences analyzed	Jurgens et al. (1997)
Arch21F / Arch915r	LMA; *Crenarchaeota* and *Euryarchaeota*	Lake Michigan sediment (0-4cm)	36 of 40 were archaeal	MacGregor et al. (1997)
1Af / 1100Ar and 1Af / 1404R	1MT, 2MT, 2C, *Euryarchaeota*	Colne Point Salt Marsh sediment (0-10cm) / Ray Creek (2C), northeast Essex, UK,	133 of 600 sequenced (length 223 bp)	Munson et al. (1997)
89Fb / Arch915R	KBS; nonthermophilic *Crenarchaeota*	Agricultural soil, Michigan	35 analyzed, 12 sequenced	Buckley et al. (1998)
ARC344 / rP2 and Arch21F / 907r	BBA, Freshwater and Marine Benthic *Crenarchaeota,* Salt Marsh and Marine Benthic *Euryarchaeota*	low-temperature anoxic marine continental shelf sediments, Buzzards Bay, Cape Cod, from the SSV Corwith Cramer (15-18cm depth in sediment)	6 sequenced	Vetriani et al. (1998)
Arch21F / Arch958R	*Crenarchaea* and *Euryarchaea*, freshwater and marine plankton: CR-PA, CRE-PA, CRE-FL, CRO	Columbia River and coastal Pacific ocean (46°15'N 124°W) near Vancouver / Portland	43 sequenced	Crump and Baross (2000)

4.2.3. More factors that influence the sequencing result

Besides the primer pairs, also the annealing temperature and the temperature sequence during the PCR (table 3) will greatly influence the final result. The addition of 5% (w/v) acetamide to a PCR mixture can minimize nonspecific annealing of the primers and prevent preferential amplification (Reysenbach et al., 1992).

Table 3. Typical PCR programs

Program	**Reference**
96℃ 90s, 60℃ 60s, 72℃ 120s / 35 cycles + final e xt step: 72℃ for 15min	Burggraf et al. (1991)
95℃ 120s, 42℃ 30s, 72℃ 240s / 25-35 cycles + fin al ext step: 72℃ for 20min	Weisburg et al. (1991)
95℃ 90s, 55℃ 90s, 72℃ 90s / 30 cycles	De Long (1992)
94℃ for 1min, 55℃ for 2min, 72℃ for 3min / over 30 cycles	Fuhrman et al. (1992)
95℃ 60s / 95℃ 60s, 55℃ 60s, 72℃ 240s / 30 cycle s + final ext step: 72℃ for 7min	Wilmotte et al. (1993)
94℃ 90s, 55℃ 90s, 72℃ 120s / 40 cycles	Barns et al. (1994)
95℃ 60s, 55℃ 60s, 72℃ 120s / 30 cycles	McInnery et al. (1995)
94℃ 4min / 94℃ 60s, 55℃ 60s, 72℃ 120s / 35 cycl es	Lee et al. (1996)
Hot start at 95℃ for 10 min and 80℃ for 2 min bef ore addition of *Taq* polymerase, followed by 10 cycles (94℃ 60s, 63℃ – 2℃/2cycles 30 s, and 72℃ 120s), then 25 cycles (92℃ 30 s; 53℃ 30 s, and 72℃ 150s), fi nal extension: 72℃ for 5 min. The very weak products of primary amplifications were pooled and concentrated to 10 µl with Micro-Con30 cartridges. Aliquots (2 µl) were used as templates for replicate (five times) secondary amplifications with the 1A and 1100R primers.	Munson et al. (1997)
94℃ for 3min / 94℃ 60s, 55℃ 30s, 72℃ 120s / 35 cycles + final ext step: 72℃ for 4min	Ronimus et al. (1997)
94℃ for 4min / 94℃ 90s, 48℃ 90s, 72℃ 120s / 30 cycles + final ext step: 72℃ for 15min	Buckley et al. (1998)
96℃ 90s / 96℃ 30s, 60℃ 30s, 72℃ 60s / 10 cycles + / 94℃ 20s, 60℃ 30s, 72℃ 60s (+ 2s for each further cycle) 25 cycles + final ext step: 72℃ for 10min	Eder et al. (1999)
96℃ 60s, 55℃ 120s, 72℃ 180s / 35 cycles	Takai & Sako (1999)
prerun 94℃ 5min / 94℃ 30s, 55℃ 30s, 72℃ 180s / 30 cycles 94℃ 10min / 95℃ 52s, 53℃-65℃ gradient 30s, 72℃ 42s	Suzuki et al. (2000)
initial 94℃ 120s / 94℃ 45s, 60℃ 45s, 72℃ 45s/ 3 0 cycles / final: 72℃ 7min	Nübel et al. (2001)
96℃ 90s / 96℃ 30s, 60℃ 30s, 72℃ 60s / 10 cycles / 94℃ 20s, 60℃ 30s, 72℃ 60s + 2s/cycle / 25 cycles / 72℃ 10 min	Rudolph et al. (2001)
96℃ 25s, 50℃ 45s, 72℃ 120s/ 30 -40 cycles	Takai et al. (2001)
Annealing temperatures between 52℃ and 61℃, 30 to 35 cycles	Casamayor et al. (2002)
94℃ 30s, 55℃ 30s, 72℃ 30s / 30 cycles	Vetriani et al. (2003)
94℃ 5min, 94℃ 60s, 45℃ 45s, 72℃ 60s / 25 cycles / final extension: 72℃ 20min 94℃ 2min, 94℃ 45s, 50℃ 45s, 72℃ 90s + 5s/cycle / 30 cycles / final extension: 72℃ 10min	Pasic et al. (2010)

During PCR two parameters can be varied, temperature and time. In the first step, the melting temperature is normally set between 94℃ an d 98℃, time is set between zero seconds and two minutes. Long, high temperature steps will damage the polymerase, they should be kept as short as necessary. The annealing temperature depends more on the primers; too low temperatures result in unspecific binding while at too high temperatures no binding will occur (Fig. 1).

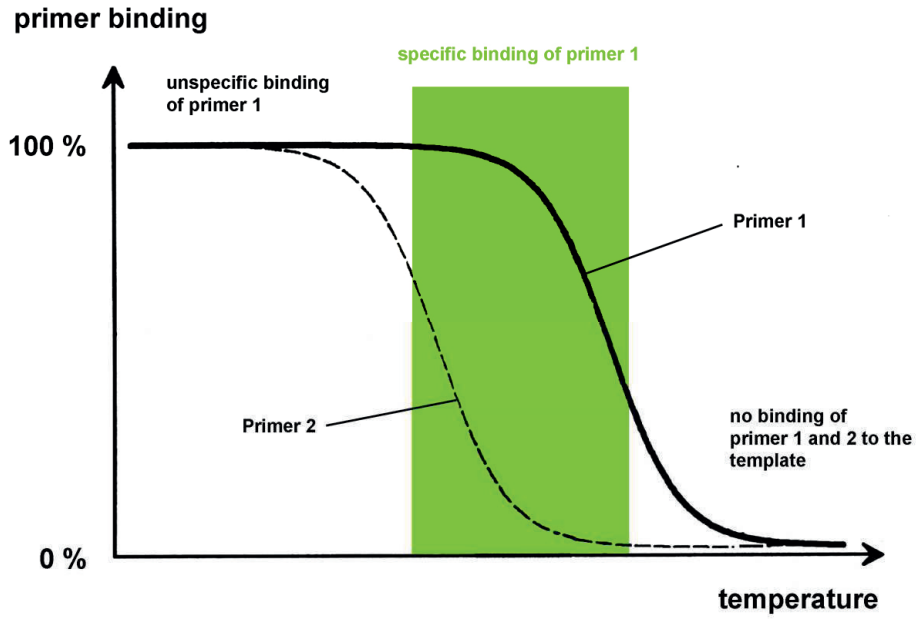

Fig. 1. Binding of two different primers to a template according to the temperature.

Nevertheless, annealing temperatures up to about 65℃ are possible, so that finally a PCR is even accomplishable holding the temperature constant at for example 62℃. The amplification temperature normally lies between 62°C to 72℃ with a duration that relates to the length of the gene and the speed of the polymerase used. *Pyrococcus furiosus* polymerase inserts up to 60 nucleotides per second and the PCR program normally is set to one to two minutes for 1000 bp at 72℃ (Gelfand & White, 1990). The *Thermus aquaticus* polymerase is slightly faster with 75 nucleotides per second (http://www.pcrstation.com/pcr-polymerases/). For "KAPA HiFi DNA Polymerase" 30 seconds are sufficient to amplify 1000 bp (www. kapabiosystems.com) and the Phusion® High-Fidelity DNA Polymerase from New England BioLabs even "runs" the 1000 basepairs in less than 15 seconds (http://www.neb.com/nebecomm/tech_reference/polymerases/phusion_high.asp). With fluorescent *in situ* hybridization, Zenklusen et al. (2008) calculated a polymerase speed in yeast of 800 +/- 70 bases per minute. Different elongation speeds have been measured in various organisms and on different genes, ranging from 0.7 kb min^{-1} to 4.4 kb min^{-1} (Ucker & Yamamoto, 1984; Edwards et al., 1991; O'Brien & Lis, 1993; Epshtein & Nudler, 2003; Mason & Struhl, 2005; Boireau et al., 2007; Darzacq et al., 2007).

The DNA extraction, the first and very important step to investigate microbial diversity, affects significantly the result, depending on the method selected (Martin-Laurent et al., 2001). At present, a lot of kits are available to extract DNA specifically from specified materials, also from the most complex environment, soil: e.g. the PowerSoil® DNA

Isolation Kit (MoBio Laboratories), the SoilMaster™ DNA extraction kit (Epicentre Biotechnologies), the FastDNA® SPIN Kit for Soil (BIO 101), the ISOIL (Nippon Gene), or the Soil DNA Isolation Kit (Norgen Biotek). All of these kits work differently. As a consequence it would be surprising to get the same DNA extract when using these kits separately on the same sample. By using a method developed individually, one knows the chemicals and concentrations used; nevertheless, it will as well deliver a different DNA composition, both qualitatively and quantitatively, compared to commercially available kits. Essentially it is impossible to find a single method that will extract completely the total DNA content from a sample. A further problem with our dolomite rock is that the binding capacity of the calcium carbonate present for DNA is high. DNA sticks to fine stone particles and precipitates during centrifugation. An extraction buffer developed for soil turned out to overcome this problem partially (Bürgmann et al., 2001; Sigler et al., 2003). Still, modifying the buffer to improve extraction and to test and compare different methods may be rewarding (Wade and Garcia-Pichel 2003). They tested three methods to extract DNA of cells from a carbonate-crust; the optimal one was based on EDTA as a chelator to dissolute the carbonate mineral and free the enclosed cells for further DNA extraction (Wade and Garcia-Pichel 2003). Stach et al. (2001) compared different extraction methods using PCR-single strand conformation polymorphism (SSCP) and experienced that environmental contaminants which were co-extracted with DNA, such as humic acids, significantly reduced primer specificity and resulted in distinct differences in sequence representation depending on the extraction methods used. Further, a greater DNA yield is not parallel to higher sequence diversity. Thus DNA extractions from soil should be evaluated not only in terms of quantity and purity, but also in terms of the sequence diversity obtained (Stach et al., 2001). Probably a combination of several different methods and pooling would collect, together as a whole, the best DNA extract from a stone sample. Still, the extraction is not the only source of bias. The following steps, such as PCR amplification, cloning, and sequencing can lead to further irregularities.

4.2.4. *Archaea* and their habitats

For 15 years after the recognition of the differences between *Archaea* and *Bacteria* in 1977 (Woese and Fox, 1977), the *Archaea* were generally known only as inhabiting hostile environments: In aquatic environments, archaeal habitats were generally limited to shallow or deep-sea anaerobic sediments with free-living and endosymbiotic methanogens, acidic hot springs or deep-sea hydrothermal vents with methanogens, sulfate reducers, and extreme thermophiles, and highly saline land-locked seas with halophiles (DeLong, 1992;

Olsen, 1994). In 1992 *Archaea* were reported for the first time to be present in temperate waters at 100m depth (14℃, 9.5x10 ^{8} cells per liter) and 500m depth (5℃, 1x10 ^{8} cells per liter) in Pacific Ocean bacterioplankton (Fuhrman et al., 1992) and a widespread occurrence of "unusual *Archaea"* in oxygenated costal surface waters of North America (Woods Hole, and Santa Barbara Channel) was described (DeLong, 1992), initiating a broad search for new environments of *Archaea*. Two years later, *Archaea* were found to cover up to 30.5% of the sub-micrometer picoplankton in the cold surface waters of Antarctica and Alaska, and in deeper waters of the North Pacific (5m and 200m depth), Santa Barbara Basin (10m and 500m depth), and Santa Barbara Channel (15m and 100m depth) (DeLong et al., 1994). Since then, *Archaea* have been detected in a variety of unexpected environments like temperate and cool ocean water (Fuhrman et al., 1997; Massana et al., 1997; DeLong et al., 1999; Murray et al., 1999; Karner et al., 2001; Lopez-Garcia et al., 2001; Eder et al., 2002; Church et al., 2003; Giovannoni and Stingl, 2005), deep marine subsurface sediments (Teske et al., 2008; Lipp et al., 2008), soil and rocks (Bintrim et al., 1997; Kemnitz et al., 2004; Kuhlman et al., 2006; Leininger et al., 2006; Walker & Pace, 2007; Valenzuela-Encinas, 2008), and also under extreme conditions as in an acid mine drainage (Baker and Banfield 2003). It has been suggested that *Archaea* may even contribute up to 20% of the total biomass on Earth, if the ocean habitat is considered to have the same domain distribution as all other habitats on Earth (DeLong and Pace, 2001). Therefore it was not surprising to find *Archaea* in alpine environments like the dolomite rock in the Piora Valley or in other environments. *Archaea* are no longer considered to be confined to extreme niche habitats (Karner et al., 2001)

4.2.5. *Archaea* and a eukaryote from the dolomite in the Piora Valley

Our search for *Archaea* yielded SSU rRNA gene sequences of four different *Crenarchaea* which had close similarities (between 98.2% and 99.9% respectively) to other already sequenced archaeal SSU rRNA genes. Interestingly we only found *Archaea* with the primer pairs 89Fb / 915R and 536f / 1392r. The primer pair 8aF / 1517r on the other hand did not yield an archaeal sequence, but a eukaryotic one: The 18S rRNA gene sequence DoAr09, which is 99.4% similar to that of *Saccamoeba limax*. Surprisingly the primer 1517r did not anneal close to the 3'-end of the gene, but about one third before at about positions 1239-1254 instead of 1891-1906. Figure 2 shows the details on the two primers 8aF and 1517r on the sequence of *Saccamoeba limax*. As indicated, Primer 1517r anneals with two or three mismatches on two positions of the gene.

Primer 8aF 5'-TCYGGTTGATCCTSCC-3' and its four different single oligonucleotides: tccggttgatcctgcc
tctggttgatcctgcc
tccggttgatcctccc
tctggttgatcctccc

Primer 1517r 5'-ATCCAGCCGCAGRTTC-3' and the reverse complement sequences to it:
gaacctgcggctggat
gaatctgcggctggat

```
DEFINITION  Saccamoeba limax small subunit ribosomal RNA gene, complete
            sequence.
ACCESSION   AF293902
SOURCE      Saccamoeba limax, strain="F-13; ATCC30942"
            Eukaryota; Amoebozoa; Tubulinea; Euamoebida; Tubulinida;
            Hartmannellidae; Saccamoeba.
REFERENCE   1  (bases 1 to 1908)
  AUTHORS   Amaral Zettler,L.A., Nerad,T.A., O'Kelly,C.J., Peglar,M.T.,
            Gillevet,P.M., Silberman,J.D. and Sogin,M.L.
  TITLE     A molecular reassessment of the Leptomyxid amoebae
  JOURNAL   Protist 151 (3), 275-282 (2000)

        1 aacctggttg atcctgccag tagtcatatg cttgtcttaa agattaagcc atgcacgtct
       61 aaggataaat atttttaac cgtaaacctg cggatggctc attaaatcag ttacaatcta
      121 ctctattgta taaattactt ggataaccgt agtaattcta gagctaatac atgcaaaaaa
      181 tttgaactcg caagggaaca aatgtattta ttaaggagca aaatcaatac agaccattcc
      241 aaactctaga ttatacttta atcggtattt tttaggggt ttgtaaacaa aaattgaaga
      301 atcatagtaa acttacgaat cataaattta attatttatt gatgatagga acgagtttct
      361 gacctattaa ctttcgatgg taatttagtg gattaccatg gttttcatgg gtaacggaga
      421 atttgggttt gattccggag aaaaggcctg agagacggcc attacttcca aggaaggcag
      481 caggcgcgta aattacccaa tcccaattcg gggaggtagt gacgagaaat actagtattg
      541 gtcccaacgg gaacaatgat tggaatgaga acaatttaaa aacattaacg actaacaatt
      601 ggagggcaag tctggtgcca gcagccgcgg taactccagc tccaagagtg tatattaaag
      661 ttgttgcggt taaaaagctc gtagttggac ttgagggatt tatataaaag gattctagtt
      721 ttaaagctag atatctttat acgtttttc ctttcttttt attttctttt aagaaggggc
      781 aactcttctg tgggaataga tagtttactt tgaaaaaatt agagtgttta aagcagacat
      841 gacatgtaca attgaatatt acagcatggg ataatagaat aggacattaa ccttaatttt
      901 gttggttttg aaggttaata gtaatgatta aaagggatag ttggggtcac ttgtatttga
      961 tcgctagggg tgaaatccta tgattgctca aagacaaact actgcgaaag catttgacaa
     1021 ggatgttttc attgatcaag aacgaaagtt aggggatcga agatgattag ataccgtcgt
     1081 agtcttaacc ataaacaatg tcgactttgg attaggaaat attacatgga tgattttcct
     1141 agcacaatat gagaaatcat aagtattcag actccggggg gagtatggtc gcaagagtga
     1201 aacttaaagg aattgacgga agggcaccac caggagtgga acctgcggct taattttgact
     1261 caacacgggg aaacttaccg ggtccggaca ctttgaggat tgacagattg atagctcttt
     1321 cttgattaag tgggtggtgg tgcatggccg tttttagttg gtggattgat ttgtctggtt
     1381 aattccgaaa acgaacgaga ccttaaccta ttaaatagta taattaaaat tgaatacaaa
     1441 ttcttgaaat ttgaaaaaaa gaactggata cttcttaaag ggactcttca tattttagtg
     1501 aatggaagtt taaggcaaaa acaggtctgt gatgccctta gatgttccgg gctgcacgcg
     1561 cgttacactg atgcgatcaa aaagtttaat ttaaatttcc ttatttgaaa agattgggta
     1621 aactcagaaa tcgtatcgtg attgggatag tgctttgtaa ttatcgcact tgaacgggga
     1681 attcctagta ggtgcaagtc atcatcttgc tccgattacg tccccgccct ttgtacacac
     1741 cgcccgtcgc tcctaccgat tgaataatcc gatgaggatt caagatagag atttcttatt
     1801 tgtctgaaag gataaaaaag aatttttta gaatctgttc aaatcttatt atttagagga
     1861 aggagaagtc gtaacaaggt ttccgtaggt gaacctgcag aaggatca
```

Fig. 2. The primers **8aF** and **1517r** and their annealing positions on the 18S rRNA gene sequence of *Saccamoeba limax*, strain F-13; ATCC30942

4.3. Microbial diversity in Piora dolomite

4.3.1. Overview

The presence of an amoeba in the dolomite rock underlines that this microcosm may be not as simple as promoted by Walker and Pace (2007a, b). A short look through the fluorescence microscope onto a DAPI treated sample of Piora dolomite material reveals a broad richness of spots of many colors (Fig. 3).

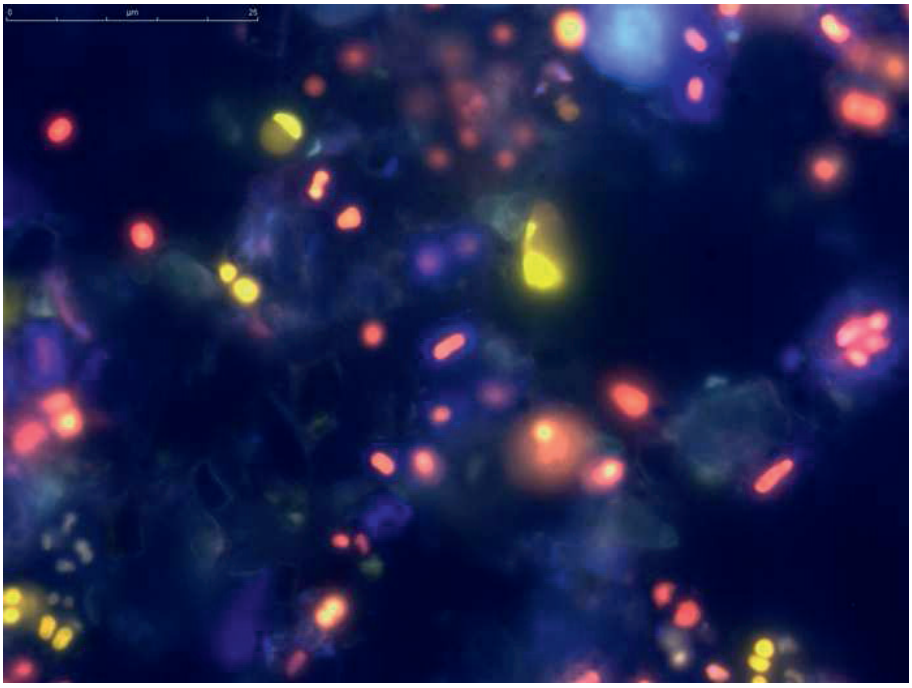

Fig. 3. DAPI stained microorganisms from dolomite rock of the Piora Valley. The different colors, shapes and structures indicate a broad diversity.

Besides *Archaea* and an amoeba as discussed before the sequencing of several clone libraries furnished a broad range of *Bacteria,* such as *Cyanobacteria*, *Actinobacteria*, *Alpha-* and *Gamma-Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Chloroflexi, Gemmatimonadetes, and Planctomycetes* and also several green algae (karyotic and chloroplastal ribosomal RNA genes). The phototrophs, predominated by *Cyanobacteria,* build the bioenergetic basis for the growth of the heterotrophic bacteria. Of special interest is the cluster found within the "phylum" uncultured *Chloroflexi,* part of the green nonsulfur bacteria. Members of the *Chloroflexi* show an extremely wide metabolic spectrum in fulfilling their energy demands. Some are strictly aerobic and chemotrophic like *Thermomicrobium*, others are anaerobic and phototrophic like the anoxygenic phototroph *Chloroflexus*. Most of them are filamentous. *Chloroflexus* contains features of the photosynthesis system of the green sulfur bacteria and of the purple bacteria. It contains bacteriochlorophyll c and chlorosomes like *Chlorobium* but the bacteriochlorophyll a located in the cytoplasmic membrane forms the photosynthetic reaction center and is

structurally identical to that of *Rhodospirillum* (Pierson & Castenholz, 1971; Pierson & Howard, 1972; Pierson, 1973). It has thus been proposed that modern *Chloroflexus* may be an offspring of a very early form of phototroph that perhaps first evolved a photosynthetic reaction center and then received chlorosome-specific genes by lateral gene transfer (Pierson & Olson, 1989; Blankenship RE, 1992; Olson & Blankenship, 2004). To characterize the photosynthetic system of the Chloroflexi-like organisms detected in the Piora Dolomite, a pure culture would be a prerequisite. With *in vivo* spectroscopy of intact rock material a shoulder at about 715 nm at the long wavelength side of the Chlorophyll a peak (680 nm) was detected (fig. 3 in Horath et al., 2006). The pigments BChl *a*, *c*, *d*, and *e* have *in vivo* absorption maxima (λ_{max}) at 830-890nm, 745-756nm, 705-740nm, and 715-726nm, respectively (Gloe et al., 1975; Jensen et al., 1964; Oelze, 1985; Scheer 2006; Kiang et al., 2007). Thus the 715 nm-peak could originate from BChl *e* (or BChl *d*) which perhaps also had been found by HPLC pigment analyses (see under "4.5.1. Absorption spectra and HPLC"). So far bacteriochlorophyll *e* has been associated only to the green sulfur bacteria, for example *Chlorobium*. The green nonsulfur bacterium *Chloroflexus* and its related phototrophs belong to one of the deepest phyla forming the only phototroph-containing branch occurring prior to the nearly simultaneous branching of all other phototrophic, bacterial phyla (Pierson, 2001). Since BChl *e* is associated with the green sulfur bacteria (GSB) and not with the green nonsulfur bacteria (GNSB), it is still open, whether a bacteriochlorophyll *e* containing Chloroflexus-like strain is dwelling in the Piora Dolomite or if the BChl *e* belongs to a green sulfur bacterium whose 16S rRNA gene sequence has not been detected so far. Interestingly the light harvesting with the chlorosomes of *Chlorobium* and *Chloroflexus* enable these organisms to grow at very low light intensities (Frigaard et al., 2004; Frigaard & Bryant, 2006), a great advantage in the habitat inside the rock.

Besides Chloroflexi-like bacteria, *Actinobacteria* were present in high amounts. This phylum is characterized by a high content of guanine and cytosine and as Gram positives they have a thicker cell wall that makes them more resistant against harsh environmental conditions. *Actinobacteria* are well represented in many endolithic habitats (table 4). Contrary to the *Actinobacteria*, the *Acidobacteria* are only sporadically present in most endolithic habitats, however, they are present in significant amounts in the Piora dolomite. One of them, clone Dolo_16, is about 94% similar to a strain of *Goodfellowia coeruleoviolacea,* and 98% similar to clone "2PJM54" which has been isolated recently from a Karstic cave in Slovenia (Pasic et al., 2010). That site consists of limestone or

dolomite and has thus a similar chemical and physical texture like Piora dolomite. It supports the idea that similar habitats may promote similar organisms. Interestingly we did not find any Chlorobi of which traces are found in the Rocky Mountain regions (Costello and Schmidt, 2006; Walker and Pace 2007a) (table 4).

4.3.2. More diversity by next generation sequencing

New methods for sequencing such as the new direct 454 sequencing are said to lower the bias between original and result of the extraction or at least of the PCR step. With 454 direct sequencing the cloning step is omitted as a source of bias. 454 sequencing first sheares the DNA sample into small fragments of 300 to 800 base-pairs. Then adapter A is ligated to one end of each fragment, and adapter B to the other. Both adapters serve later as primer sites during emulsion PCR (emPCR) and adapter A during sequencing PCR. Adapter B additionally contains a 5'-biotin tag to dock onto a streptavidin-coated bead. The bead is already covered with a lot of plus strands of B-adapters. The fragments are molten into single strand DNA and each bead gets one plus-strand fragment with the two adapters ligated to it. Then the beads are put into an emulsion bubble and PCR is performed in it. This way the special fragment becomes amplified without contamination and each new minus strand is attached to one of the other plus strand-B-adaptors on the bead. Finally there is a bead with one plus strand and many minus strands of the fragment on it. The beads then are separated from the emulsion bubble and transferred into a mini-Micro Titer Plate, the Pico Titer Plate, each bead in a separate well. The sequencing is performed in the Pico-titer plate. Per well also smaller beads are added that contain the enzymes for sequencing. Sequencing is accomplished by synthesizing the complementary strands (plus strands) of the templates attached to the bead. In a number of cycles the four bases (A, T, G, and C) are sequentially washed over the Pico Titer Plate. The incorporation of a new base is associated with the release of inorganic pyrophosphate starting a chemical cascade. This results in the generation of a light signal which is captured by a CCD camera. According to the light flashes at the respective "base washings" the sequence in each well is constructed (Chi, 2008; http://en.wikipedia.org/wiki/454_Life_Sciences; http://www.youtube.com/watch?v=bFNjxKHP8Jc; http://www.dkfz.de/gpcf/242.html). This method of sequencing can handle much higher amounts of reads and outcompetes the classic Sanger method in spite of that it reads only small fragments because of the big amount of reads in parallel. Once the sequences of the fragments are read, they need to be assembled to the original full length. This might be a drawback as the assembly of repetitive regions is difficult. Normally a sequencing of a sample therefore needs multiple

coverage of several times. Nevertheless, also with the 454 sequencing it is not possible to get the complete meta-genome of a prokaryotic community because for example the DNA of some cells is not extracted or other cells get strongly broken so that their DNA gets destroyed as well.

On table 4 several studies on endolithic microorganisms are compiled giving the percental composition of the different phyla according to the SSU rRNA genes sequenced in each work. An accurate comparison is not possible since different DNA extractions, different primers, and different DNA extraction methods have been used. Sometimes several experiments from one location have been combined, as indicated in the list of primers used. More than one primer pair indicates multiple PCR experiments. The dolomite community from Piora resembles most to the community of the McMurdo Dry Valleys in Antarctica. Thereby it may be of interest that the McMurdo Dry Valleys are oriented west-to-east like the Piora Valley. The Dry Valleys soil ecosystems are characterized by large variations in temperature (Vincent WF, 1988; Doran et al., 2002; Aislabie et al., 2006) and also the air temperatures of the rock in the Piora Valley oscillate between -18° and 30℃ during winter and summer (Fig. 5). However, the temperature in a rock cavity stayed between -5° and 17℃. As mentioned before, the pred ominance of the *Actinobacteria* in most endolithic habitats is remarkable besides the huge group of the *Proteobacteria*. It is probably related to their strong cell wall, the high GC content, and the ability of some of its genera to form spores. By forming spores they enter a dormancy state and thereby overcome unfavorable environmental conditions. Microorganisms entering a reversible state of low metabolic activity become part of a seed bank, which contributes to the maintenance of microbial diversity in the specific environment (Jones and Lennon, 2010). Besides *Proteobacteria*, green algae, *Actinobacteria*, *Cyanobacteria*, *Crenarchaea*, *Bacteroidetes*, and *Firmicutes* (Low GC Gram positive bacteria), also the *Acidobacteria* are on average with 4.0 % well represented in the endolithic environment.
Bryant et al. (2007) analyzed metagenomic data obtained from phototrophic mat communities in the Yellowstone National Park and detected genes of the probable 5'-pscA-pscB-fmoA-3' operon and genes flanking that operon that were most similar to those of "*Candidatus* Koribacter versatilis Ellin345" or "*Candidatus* Solibacter usitatus Ellin6076", two soil bacteria belonging to the phylum *Acidobacteria*. The genes pscA, pscB, and fmoA are coding for a bacteriochlorophyll a-binding apoprotein of a homodimeric, P840-binding, type 1 reaction center, an apoprotein of an 8Fe-8S ferredoxin of a type 1 reaction center, and a bacteriochlorophyll a-binding, the Fenna-Matthews-Olson protein, respectively. So it

Table 4. Comparison of different endolithic environments by analysis of sequences of the small subunit ribosomal RNA gene

Reference	M.C.Smith et al., 2000	M.C.Smith et al., 2000	de la Torre et al. (1+2)/2, 2003	Barton et al., 2004	Walker et al., 2005	McNamara et al., 2006	Kuhlman et al., 2006	Costello & Schmidt 2006	Nemergut et al., 2007	Walker & Pace, 2007a	Dong et al., 2007	Horath & Bachofen, 2009	
sample source	sublithic quartz, Antarctica, cultivated	sublithic quartz, Antarctica, clones	Beacon sandstone from the McMurdo Dry Valleys, Antarctica	wall in Fairy Cave, Colorado	geothermal rock of Yellowstone (pH 1)	limestone, Ek Balam, Yucatan, Mexico	rock varnish, Whipple Mountains, Arizona	alpine tundra wet meadow soil (0–20 cm) Rocky Mountains	glacier forelands, Peruvian Andes	Rocky Mountains	Atacama Desert, (colored + control) /2	Piora Dolomite	Average
Alpha Proteobacteria	0.135	0.030	0.180	0.218	0.010	0.042	0.184	0.084	0.029	0.094	0.267	0.028	0.108
Beta Proteobacteria	0.120			0.090	0.010	0.021	0.026	0.071	0.647	0.014			0.083
Gamma Proteobacteria	0.130	0.010	0.010	0.038	0.090	0.063		0.032	0.036	0.003	0.105	0.020	0.045
Delta Proteobacteria						0.021	0.053	0.026		0.008	0.019		0.011
Bacteroidetes (=CFB)	0.225	0.080	0.038	0.051	0.070			0.091	0.050	0.061	0.048	0.048	0.064
Chlorobi								0.010		0.001			0.001
Gemmatimonadetes						0.042		0.026		0.006	0.057	0.008	0.012
Verrucomicrobia								0.065		0.009	0.029		0.009
Planctomycetes		0.020	0.010					0.026		0.003	0.019	0.004	0.007
Nitrospira					0.010			0.026					0.003
Acido-bacteria			0.005			0.147		0.240	0.007	0.019		0.056	0.040
Cyano-bacteria	0.100	0.680	0.165					0.006	0.165	0.271	0.238	0.227	0.154
Firmicutes (Low GC)			0.008	0.372	0.020	0.213				0.024	0.114		0.063
Actinobacteria (high GC gram +)	0.290	0.180	0.055	0.231	0.480	0.443	0.132	0.032	0.007	0.240	0.105	0.060	0.188
Chloroflexi / GNS			0.010				0.053	0.149		0.031		0.024	0.022
Thermus / Deinococcus			0.130						0.014	0.013			0.013
TM7							0.026	0.006				0.008	0.003
OP10								0.019		0.007			0.002
Cren-archaeota		nd		0.000	0.010	nd	0.105	0.084		0.077	nd	0.159	0.073
Eury-archaeota		nd		0.000	0.040	nd		0.000			nd		0.013
Fungi & Chlorophyta & Rhodophyta & Chloroplast of Diatoms (Eukarya)		0.005	0.400	nd	0.260	nd	0.421	0.006	0.043	0.121	nd	0.359	0.202
Total	1.000	1.005	1.011	1.000	1.000	0.992	1.000	0.999	0.998	1.002	1.001	1.001	1.001
primers used	530f, 1492r (?)	530f, 1492r	27F, 1391R 27F, 1492R 333Fa, 1391R 515F, 1391R 515F, 1492R 82Fe, 1391R 356Fcya,1391R 21Fa, 958Ra	27F, 805R 333Fa, 1391R	27F, 1391R 515F, 1391R	341f, 907r	338f,907r A21f, A958r NS3, NS8 (eukaryotic)	27F, 1391R 8Fa, 1492R	530f, 1492r	27F, 1391R 515F, 1391R	27f, Univ1492	27f, 1524r 8aF, 1517r 536f, 1392r 8aF, 1512uR 89Fb, 915R CYA359F, CYA1342R	

is possible that there might also be phototrophic *Acidobacteria* dwelling in the Piora Dolomite. So far five bacterial phyla contain chlorophototrophs: *Cyanobacteria*, *Chlorobi*, *Proteobacteria*, *Chloroflexi*, and *Firmicutes* (with the *Heliobacteria*). The *Acidobacteria* would provide the sixth phylum that contains phototrophic species. Thus the ability to form spores or dormant cells, to have a thick cell wall, and / or to perform photosynthesis provides organisms with an advantage to survive at the harsh conditions in the high mountains or the Arctic endolithic environment.

4.4. Environmental factors governing endolithic organisms

4.4.1. Temperature measurement

On our sampling spot several temperature measurements were performed, demonstrating that the stone can store a considerable amount of heat energy.

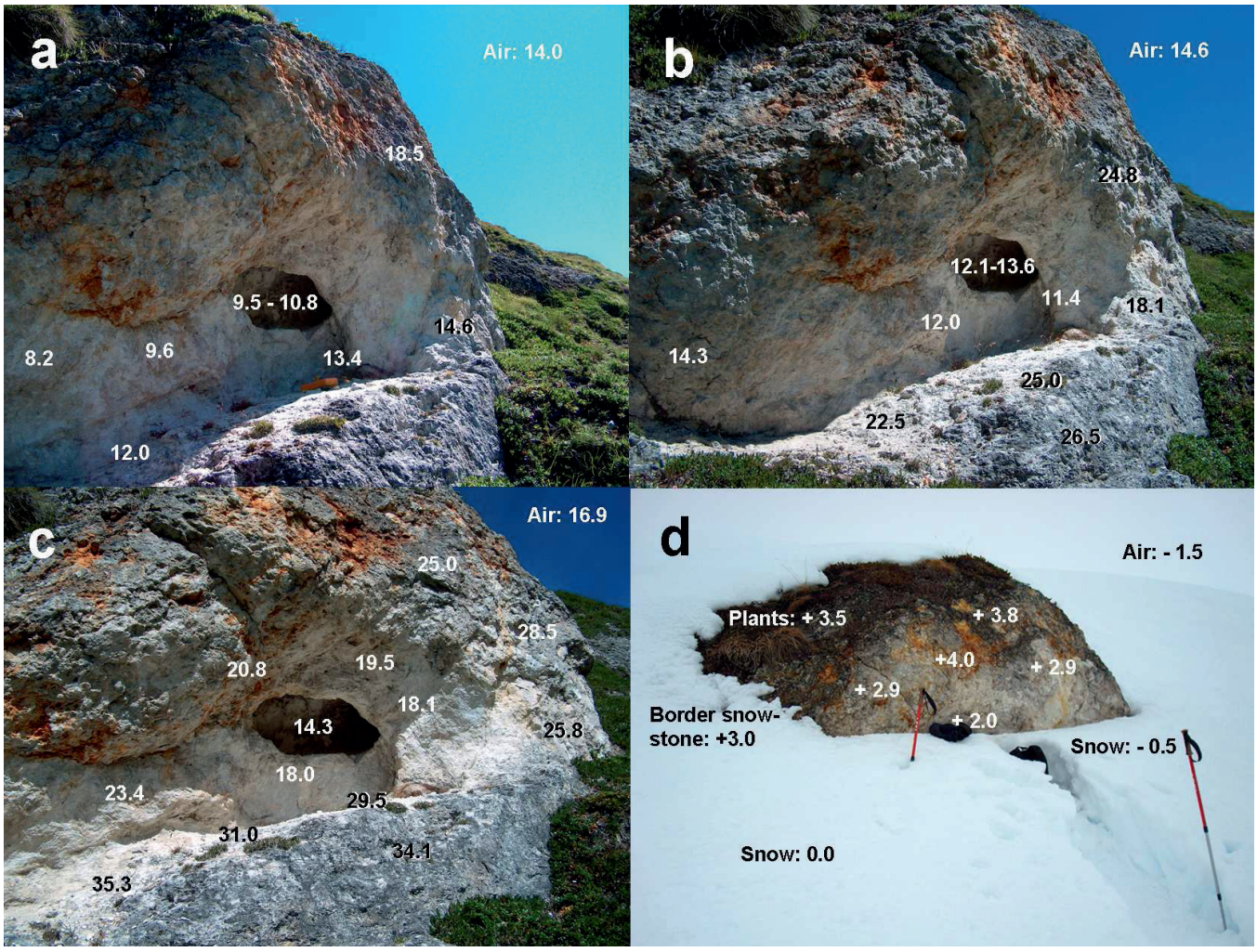

Fig. 4. Surface temperatures [℃] measured with an infrare d radiation thermometer (IR-352 by Voltcraft ®, Germany) at sampling location on a) 15.8.2004, 11h15; b) 13h15; c) 15h15; and d) on 20.3.2010, 14h30.

On a sunny day in August, the surface temperature of the rock may differ from the air temperature by more than 20℃, depending on the exposure of the rock wall towards the sun (Fig. 4). On a single measurement in March 20 of 2010, a cloudy and foggy day, a rock embedded in snow was warmer than the surrounding air and ground (Fig. 4d). A temperature data logger placed in the cavity illustrated in Fig. 4 a, b, c between end of October 2007 to end of July 2008 illustrates the temperature variation in the cavity over one year. The temperature was registered every hour (Fig. 5 with details in figs. 6 and 7). Compared with the course of temperature in Piotta which has been corrected to the altitude of Cadagno ("T [℃] (at (a + x) meters altitude) = T [℃] (at a meters altitude) – [(x meters / 1000 meters) * 6.5℃]"), the temperature in the cavity of the rock is more damped than the one 2 m above ground or 5 cm above grass. During December and January the temperature in the rock cavity is on average even slightly higher than the real temperature down in Piotta. This can be explained on one hand with a temperature inversion in the valley which forms in the winter when thick fog prevents the sun from warming up the ground. On the other hand, also a thick cover of snow insulates from large temperature oscillations. One might not expect that the conditions are more harsh down in the valley than up in the mountain in a rock cavity. A comparison with summer temperatures obtained from soil measurements in the Antarctica shows that similar temperature oscillations are found there as well (Aislabie et al., 2006) (Fig. 8).

In the Dry Valleys of Antarctica, air and soil temperatures are highly variable. Depending on aspect, altitude, and other topographical factors, the mean annual temperatures range from -15℃ to -30℃ (Doran et al ., 2002; Aislabie et al., 2006). However, the soils of the surface are subject to larger daily temperature fluctuations during the austral summer of 25–75 days with temperatures above 0℃ and liquid water available (Doran et al., 2002; Barrett et al., 2008b). Maximum temperatures are typically in the range of +17℃ to +26℃, and daily temperature fluctuations of more than 20℃ are not unusual, often resulting in multiple freeze–thaw cycles in a single day (Aislabie et al., 2006; Barrett et al., 2008a). In the winter months, minimum temperatures typically range from -40℃ to -60℃ (Cary et al., 2010). This is clearly much lower than in the Dolomite rock of the Piora Valley. Normally a one to two meter thick snow layer covers the rock wall in the Piora Valley during the winter (see fig. 4d) which provides a good insulation against strong amplitudes of temperature. In the Dry Valleys of Antarctica, snow covers may get blown away resulting in a less buffered temperature oscillation.

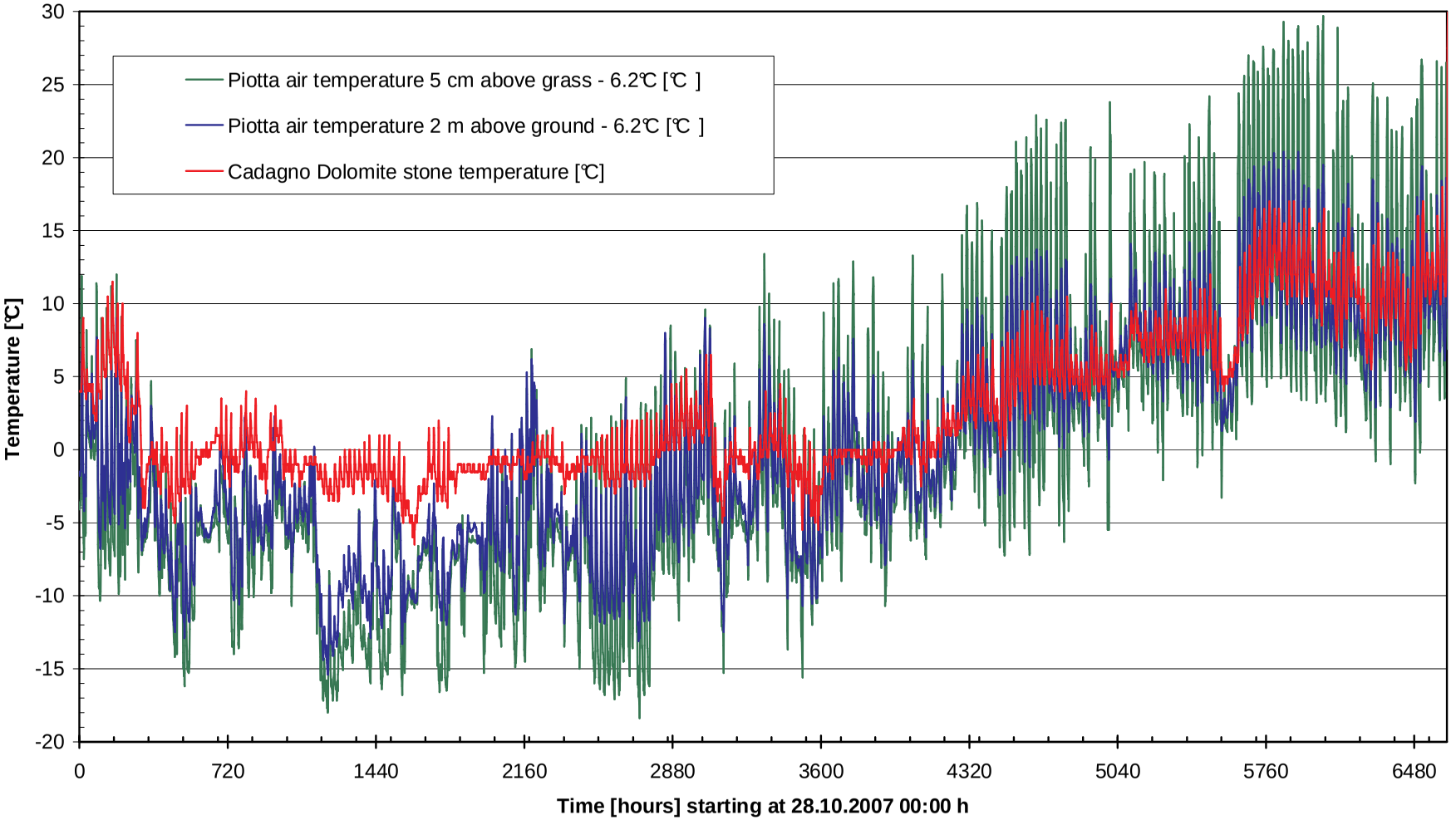

Fig. 5. Temperature measurement in the cavity of the sampled rock in the Piora Valley (46°32'51"N 8°43'05"E; r ed line) and altitude corrected values (- 6.5℃ / 1000m higher) from Piotta (46°31'02"N 8°40'32"E) du ring the same time 2m above ground (blue line) and 5 cm above grass (green line) from 28.10.2007 0h00 till 31.7.2008 14h00 (=6638h).

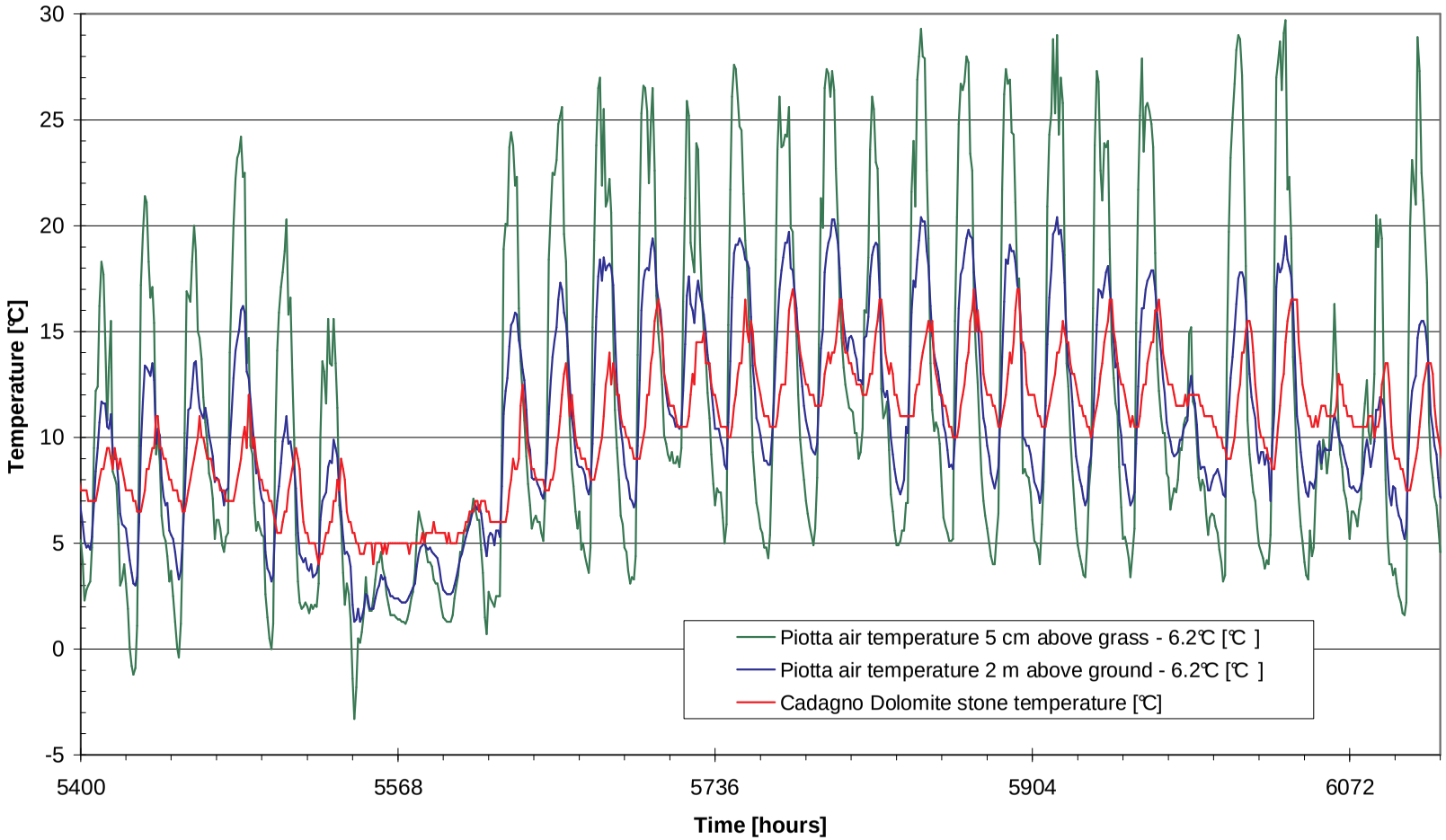

Fig. 6. Detail of figure 5 in summer from 9th of June 00h00 to 8th of July 2008, 23h59. The daily peaks are smaller in the rock (red) compared to the air temperature both 2m above ground (blue) and 5 cm above grass (green) in Piotta.

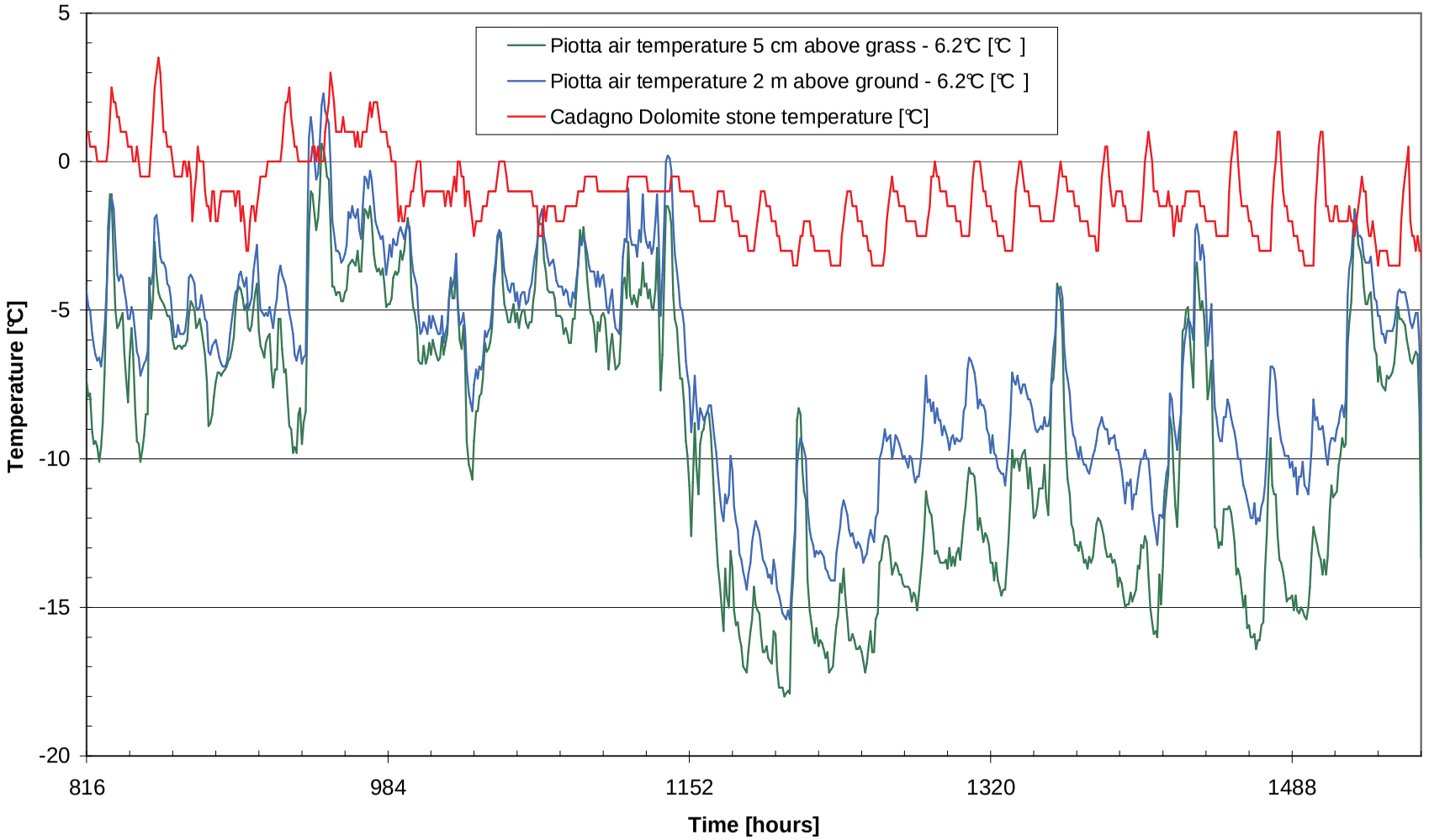

Fig. 7. Detail of figure 5 in winter between 1st of December 00h00 and 31st of December 2007, 23h59. Temperature in the rock cavity at Cadagno and in Piotta (-6.2℃). The daily peaks are smaller in the rock cavity than in the valley at Piotta.

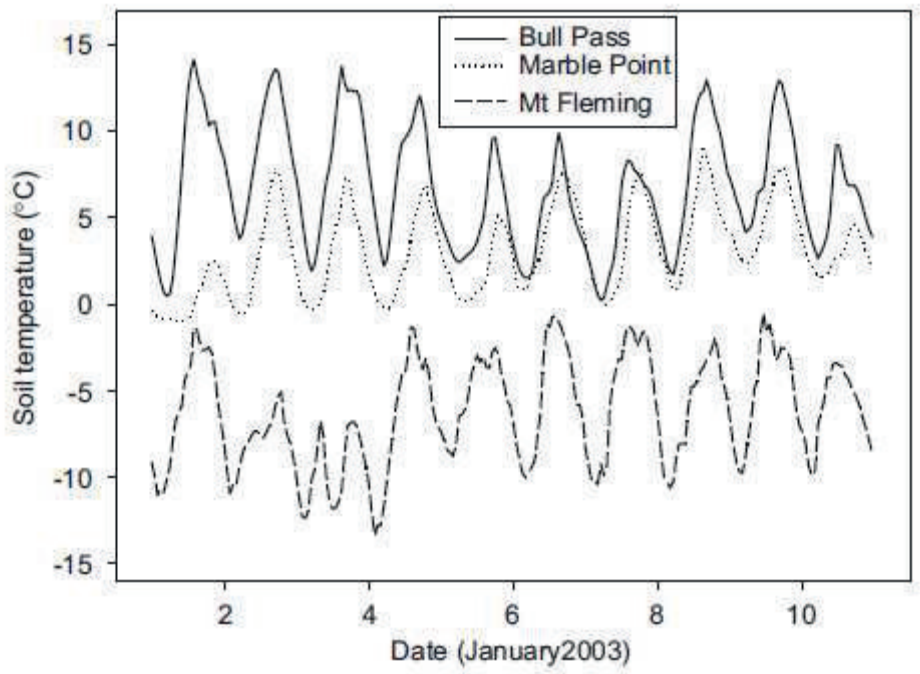

Fig. 8. Temperature of soil at 2 cm depth measured for 10 days in the Antarctic summer from three locations of the Antarctica: Bull Pass 77°31'S 161°52'E (betw een Marble Point and Mount Fleming), Marble Point (close to Pacific coast) 77°25'S 163°41'E, and Moun t Fleming 77°33'S 160°17'E (ca. 87 km away from the coast towards heartland) (Aislabie et al., 2006).

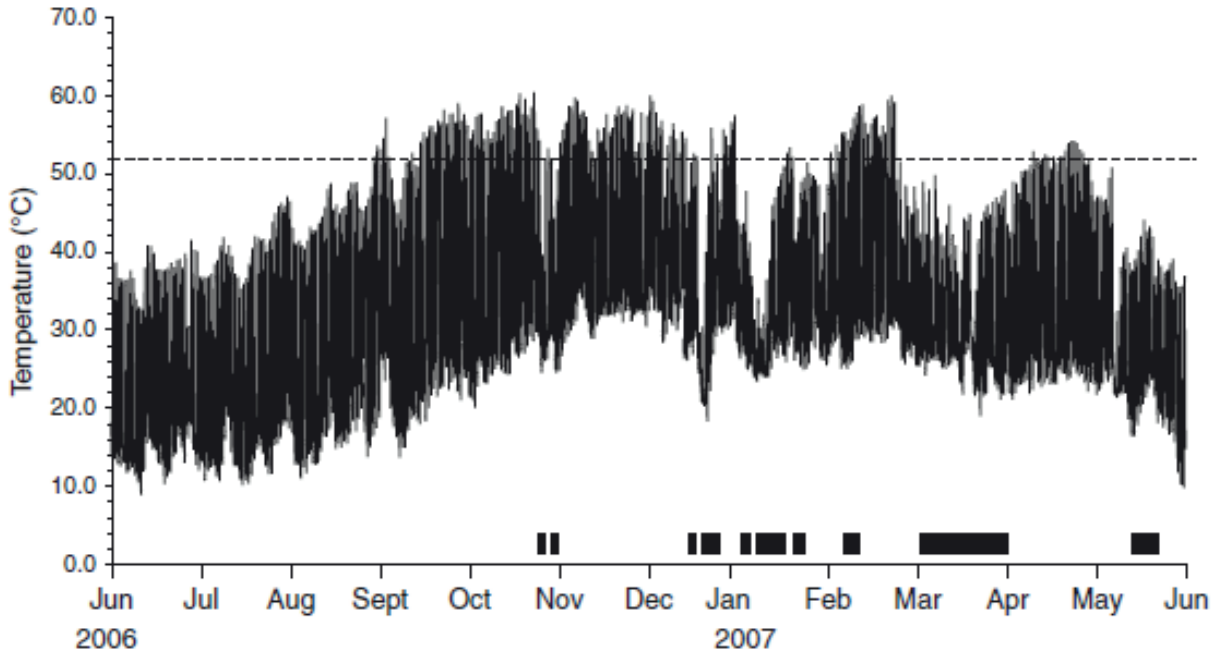

Fig. 9. Microclimate temperature of a semiarid region of tropical Australia under translucent rocks that supported hypolithic cyanobacteria (ca. 17°34'S 130°52'E). Data are means of hourly temperatures recor ded below several rocks, between 3rd of June 2006 and 10th of June 2007. The broken line represents the maximum temperature for photosynthesis by cyanobacterial communities. Black bars at the bottom of the graph indicate days when the soil water content was above 0.15 m^3 m^{-3}, the threshold for photosynthetic activity of hypolithic communities (Tracy et al., 2010).

4.4.2. Water loss

Almost the opposite side of the temperature spectrum is found in the semiarid region of tropical Australia, where cyanobacteria grow below translucent rocks. There the temperature does not drop below about +10℃ and can reach up to about +60℃ (Tracy et al., 2010) (Fig. 9). In both cases one of the strategies for surviving these harsh conditions is drying out. For rehydration, the microorganisms are well prepared; endoliths take up water and swell rapidly. Sometimes even the humidity in the air is sufficient, as endolithically living lichens solely utilize water vapor to achieve positive net photosynthesis rates (Weber et al., 2007).

4.5. Diversity of photosynthetic pigments

4.5.1. Absorption spectra and HPLC

The methanol extract of a sample of the greenish band of a dolomite stone resulted in an absorption spectrum with maxima at 207, 267, 313, 387 (shoulder), 414 (shoulder), 436, 467, 612, and 664 nm wavelength (Fig. 5a in Horath et al., 2006). According to Namsaraev (2009) the absorption in methanol at 664 nm is most probably due to chlorophyll *a* (maximal absorption in methanol at λ_{max} =665 nm) but bacteriochlorophyll *c* ($\lambda_{max,methanol}$ = 667 nm) also absorbs in this range. Thus the methanolic extract of a Piora dolomite is dominated by the absorption spectrum of chlorophyll a, the main pigment in cyanobacteria, algae, and plants. High pressure liquid chromatography (HPLC) combined with a photo diode array detector allowed to separate a diversity of pigments. The strongest absorption peaks appeared after about 14 and 28 minutes (Fig.10).

The pigments after 14 minutes absorbed between 350 nm and 450 nm and are likely attributed to scytonemin (Fig. 5.b in Horath et al., 2006). Scytonemin absorbs strongly in the UV-A (315-400 nm), the spectral region of 325–425 nm, with an *in vivo* maximum at 370 nm (Garcia-Pichel & Castenholz, 1991; Proteau et al., 1993). The spectrum obtained from Piora dolomite (minute 14.3) corresponds to the one of Proteau et al. (1993) and Singh et al. (2009) (Fig.11). Scytonemin is well known as protecting against high light conditions. The eluate at 28.3 minutes, the second most prominent peak, is chlorophyll *a* with absorption maxima at 431 nm and 664 nm (Fig.5h in Horath et al., 2006). Bacteriochlorophyll *e* perhaps was present in trace amounts. According to Borrego et al. (1999), bacteriochlorophyll *e* has an absorption maximum between 649 and 660 nm depending on the solvent used. The compound eluting at 23.3 minutes with maxima at 466 nm, 601 nm, and 650 nm could be allocated to bacteriochlorophyll *e*, since absorption maxima at 466 nm and 651 nm are typical for BChl *e* in aceton:methanol (7:2) (Borrego et

al., 1999). Difficulties in allocation arise due to shifts of the peaks depending on the polarity of the solvent used. BChl *e* has the maxima at 462 nm and 649 nm in acetone, but 476 nm and 660 nm in methanol (Borrego et al., 1999).

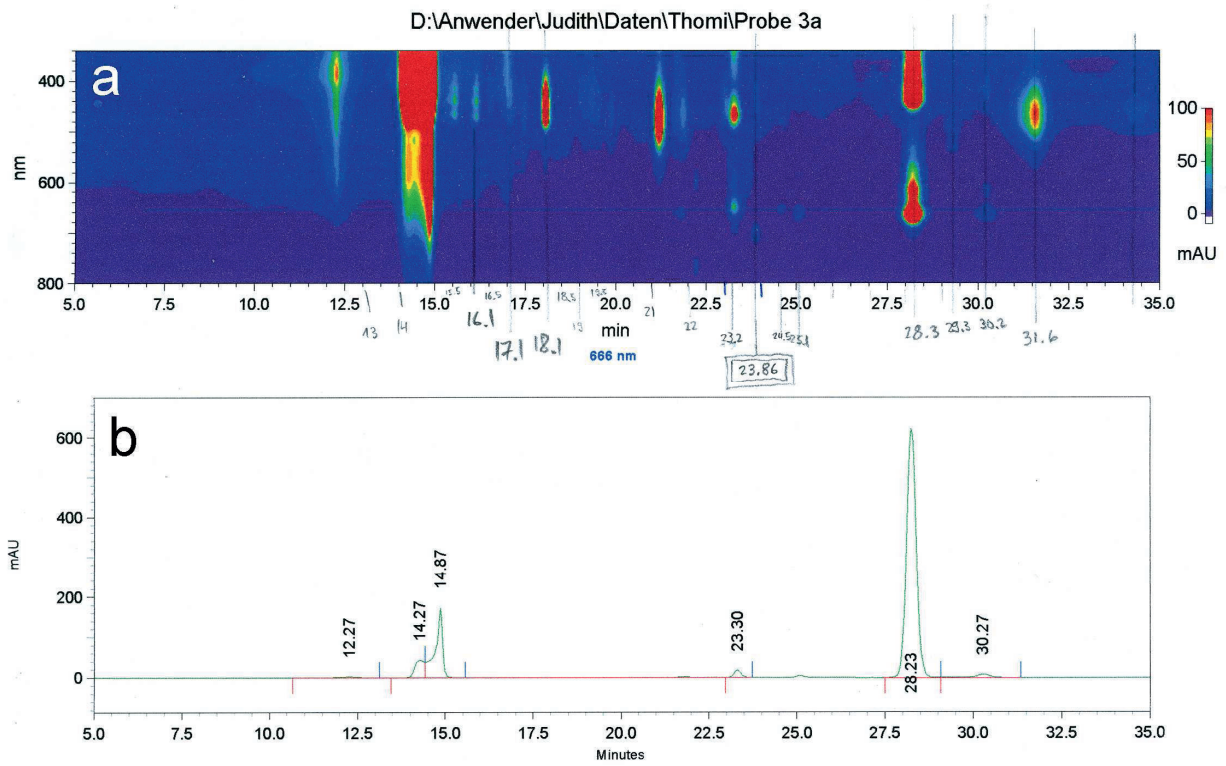

Fig. 10. Contour plot (a) and chromatogramm at 650 nm (b) of dolomite extract after separation by HPLC (C-18 RP)

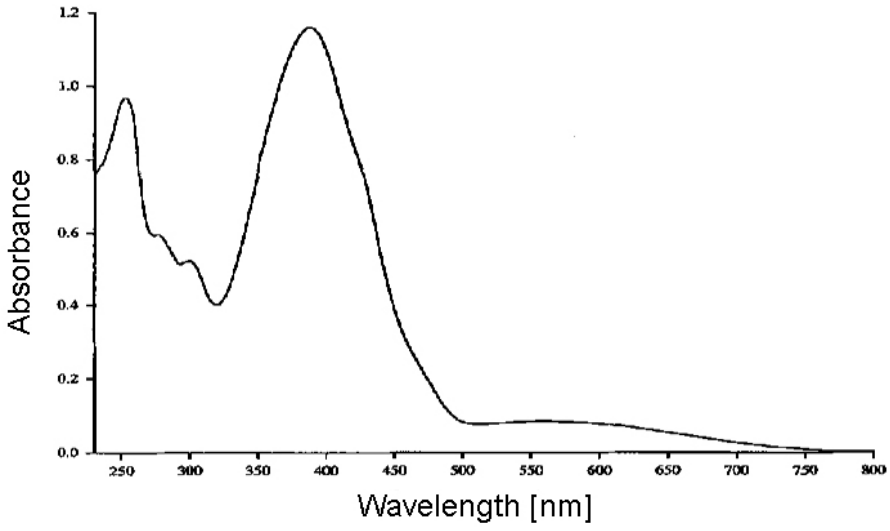

Fig. 11. Absorption spectrum of scytonemin in tetrahydrofuran with peaks at 252, 278, 300 and 386 nm (Proteau et al., 1993; Singh et al., 2009)

Several absorption peaks remained unidentified and are waiting for further investigation. This holds especially for the spectra that eluted at 21.23, 21.73, 23.93, and 30.27 minutes,

shown in figures 12 to 14. These eluates all have maxima that suggest a kind of chlorophyll but are difficult to allocate to any known (bacterio-) chlorophylll. Their long wavelength λ_{max} lie at 663 nm (fig. 12 and fig.14), and 706 nm (fig. 13). The eluate at minute 23.87 is of special interest, since its Qy band absorbs at a wavelength above 700 nm, typical for a bacteriochlorophyll. The addition of 10% 1M ammonium acetate to the sample as an ion pairing agent to improve the separation and resolution of the bacteriochlorophyll homologues (Borrego et al., 1999) perhaps could help to detect the different bacteriochlorophylls. The correct allocation of maximum absorption peaks of eluates of a gradient-HPLC is not simple since the eluent polarity is constantly changing.

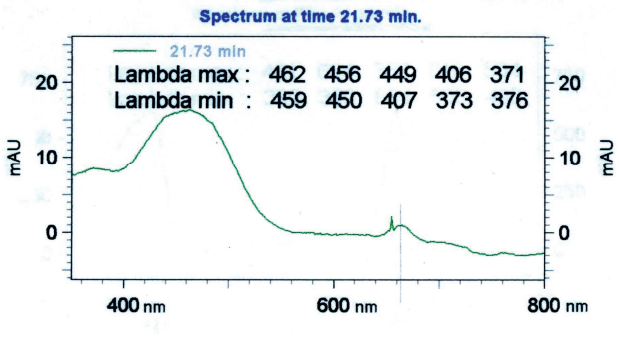

Fig. 12. Absorption spectrum of the eluate of dolomite extract in methanol:acetone of about (8:2) eluting after minute 21.73.

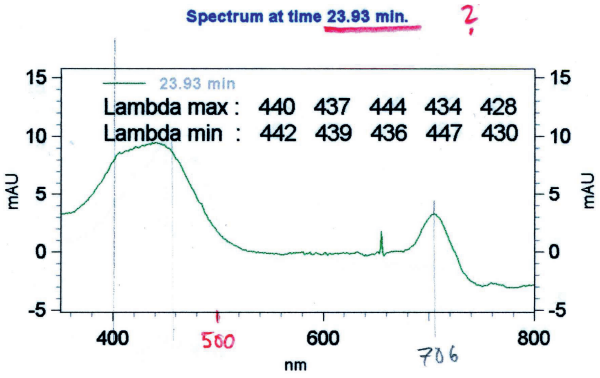

Fig. 13. Absorption spectrum of the eluate of dolomite extract in methanol:acetone of about (8:2) eluting after minute. 23.93.

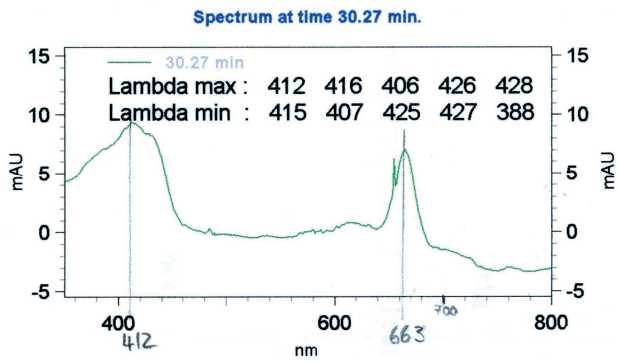

Fig. 14. Absorption spectrum of the eluate of dolomite extract in methanol:acetone of about (8:2) eluting after minute 30.27.

For comparison and help for identification, the λ_{max} for the two main absorption bands of chlorophyll *a* and *b* and the known bacteriochlorophyll pigments in different solvents are listed in table 5 (modified after Namsaraev, 2009). This illustrates that small variations of λ_{max} are due to the composition of the solvent, the purity of the preparation and are possibly also dependent on the strain used to extract the specific chlorophyll. Thus the wavelengths of maximal absorption are not sufficient to fully determine the chlorophyll species.

Table 5. Soret and Q_y band position in nanometers for different pigments and solvents. Background color is used to better distinguish between different chlorophylls. Modified, after Namsaraev (2009)

Pigment	**Solvent**	**Soret band [nm]**	**Q_y band [nm]**	**Reference**
Chl a	acetone:water (8:2)		663.6	Porra et al., 1989
Chl a	acetone:water (8:2)	431.2	663.2	Lichtenthaler, 1987
Chl a	acetone	429.6	661.6	Lichtenthaler, 1987
Chl a	acetone	430	663	MacKinney, 1940
Chl a	acetone	430.3	662.1	SCOR WG 78 data
Chl a	acetone	430.1	662.0	Seely & Jensen, 1965
Chl a	acetone		662.7	Jeffrey & Humphrey, 1975
Chl a	methanol	431.8	665.2	Lichtenthaler, 1987
Chl a	methanol	432.0	665.7	Seely & Jensen, 1965
Chl a	acetone:water (8:2)	433	665	Vernon, 1960
Chl a	diethyl ether	429	660.8	Porra et al., 1989
Chl a	ether	430	662	Goedheer, 1966
Chl a	HPLC eluent [1)]	430	663	Frigaard et al., 1996
Chl a	*in vivo*	425	680	Goedheer, 1966
Chl a	*in vivo*	436	673-680	Sathyendranath et al., 1987
Chl a	*in vivo*	440	677	Faust & Norris, 1982 (*Anabaena flos-aquae)*
Chl b	acetone	455	645	MacKinney, 1940
Chl b	acetone	455.8	644.8	Lichtenthaler, 1987
Chl b	acetone		647	Vernon, 1960
Chl b	acetone	456.9	645.5	SCOR WG 78 data
Chl b	acetone:water (8:2)		646.6	Porra et al., 1989
Chl b	acetone:water (9:1)		646.8	Jeffrey & Humphrey, 1975
Chl b	acetone:water (8:2)	460	648-649	Vernon, 1960
Chl b	acetone:water (8:2)	459.0	646.8	Lichtenthaler, 1987
Chl b	methanol		652.0	Porra et al., 1989
Chl b	methanol	469.2	652.4	Lichtenthaler, 1987

Table 5 (continued)

Pigment	Solvent	Soret band [nm]	Q_y band [nm]	Reference
Chl b	diethyl ether	454	643	Porra et al., 1989
Chl b	HPLC eluent [1)]	463	648	Frigaard et al., 1996
Chl b	*in vivo*	470-480	650	Hoepffner & Sathyendranath, 1992
BChl a	acetone		768-770	Sauer et al., 1966; Connolly et al., 1982; Korthals & Steenbergen, 1985
BChl a	acetone		770.6	Permentier et al., 2000
BChl a	acetone	358	770-773	Jensen et al., 1964
BChl a	acetone	359	770	Pierson & Castenholz, 1974 *(Chloroflexus aurantiacus)*
BChl a	acetone:methanol (7:2)	ca 368	ca 770	Oelze, 1985 (*Rhodopseudomonas sphaeroides*)
BChl a	methanol		771.0	Permentier et al., 2000
BChl a	methanol	365	772	Smith & Benitez, 1955 (Rhodospirillum rubrum)
BChl a	methanol	366	771	Sistrom, 1966 (Rhodopseudomonas sphaeroides Ga)
BChl a	HPLC eluent [1)]	364	770	Frigaard et al., 1996
BChl a	*in vivo*	258, 375	805, 850	Jensen et al., 1964
BChl a	*in vivo*	375	802, 866	Feick et al., 1982
BChl b	ethyl ether	372, 406	791	Oelze, 1985 (Rhodopseudomonas viridis)
BChl b	HPLC eluent [1)]	373	795	Frigaard et al., 1996
BChl b	acetone	368, 407	795	Jensen et al., 1964
BChl b	acetone	368, 407	794	Eimhjellen et al., 1963
BChl b	*in vivo*	400	843, 1020	Jensen et al., 1964
BChl b	*in vivo*	400	606, 1020	Drews & Giesbrecht, 1966
BChl c	acetone	433	662.5	Stanier & Smith 1960, Oelze, 1985
BChl c	acetone	435	667	Maresca et al., 2004
BChl c	acetone	428	660	Gloe et al., 1975
BChl c	acetone	433	662	Pierson & Castenholz, 1974 *(Chloroflexus aurantiacus)*
BChl c	acetone	434	663	Pierson & Castenholz, 1974 *(Chlorobium limicola)*
BChl c	acetone:methanol (7:2)		666	Feick et al., 1982
BChl c	methanol	435	670	Stanier & Smith, 1960
BChl c	methanol	435	668-669	Pierson & Castenholz, 1974 *(Chloroflexus aurantiacus)*

Table 5 (continued)

Pigment	Solvent	Soret band [nm]	Q_y band [nm]	Reference
BChl c	methanol	435	669	Pierson & Castenholz, 1974 *(Chlorobium limicola)*
BChl c	methanol		667	Maresca et al., 2004
BChl c	HPLC eluent [1)]	434	666	Frigaard et al., 1996
BChl c	*in vivo*		746	Holt, 1966
BChl c	*in vivo*	460-462	750-751	Maresca et al., 2004
BChl c	*in vivo*	460	740	Feick et al., 1982
BChl c	*in vivo*	457	756	Gloe et al., 1975 (*Chlorobium limicola forma thiosulfatophilum)*
BChl d	acetone	427	654	Stanier & Smith, 1960; Oelze, 1985
BChl d	acetone	428	655-656	Maresca et al., 2004
BChl d	acetone	424	654	Gloe et al., 1975
BChl d	methanol	427	659	Stanier & Smith, 1960
BChl d	methanol		657	Maresca et al., 2004
BChl d	HPLC eluent [1)]	427	655	Frigaard et al., 1996
BChl d	*in vivo*		725	Holt, 1966
BChl d	*in vivo*	446-451	733-736	Maresca et al., 2004
BChl d	*in vivo*	445-452	725-736	Gloe et al., 1975
BChl e	acetone	462	649	Borrego et al 1999
BChl e	acetone	456-459	646-648	Gloe et al., 1975
BChl e	acetone:methanol (7:2)	466	651	Borrego et al., 1999
BChl e	methanol	476	660	Borrego et al., 1999
BChl e	HPLC eluent [1)]	469	654	Frigaard et al., 1996
BChl e	*in vivo*	452-460	715-726	Gloe et al., 1975
BChl g	acetone		761.6	Van de Meent et al., 1991
BChl g	acetone?	418	763	Scheer 1991
BChl g	acetone	365 405	762	Scheer 2006
BChl g	dioxane	408, 418	763	Brockmann & Lipinski 1983
BChl g	*in vivo*	372, 421	788	Gest & Favinger 1983 of *Heliobacterium chlorum*
BChl g	*in vivo*	412	788	Fuller et al., 1985 of *Heliobacterium chlorum*

[1)] The gradient of the HPLC was composed of solvent A (methanol:acetonitrile:water, 42:33:25 by volume) and solvent B (methanol:acetonitrile:ethyl acetate, 39:31:30 by volume).

4.5.2. *In situ* reflectance spectroscopy

The *in vivo* reflectance spectroscopy as a transect across the greenish endolithic bands resulted in a prominent peak at around 680 nm, which is typical for chlorophyll *a*. Comparing spectra from slightly different spots some differences were detected (Fig.15). The deeper inwards the rock the detection went, the more the absorption in the range of 570 to 650 nm increased, due to a higher concentration of phycobilins, especially phycocyanins. Phycoerythrin and phycocyanin are produced by *Cyanobacteria* to use a bigger part of the sunlight spectrum for photosynthesis. The accessory pigments collect light in the range of about 480 to 570 nm and 570 to 650 nm respectively (Kronick, 1986) and transfer the energy to the two Chl *a* in the photosynthetic reaction center. This so called chromatic adaptation in *Cyanobacteria* enables them to grow at fairly low light intensities. Indeed the darker the conditions for the *Cyanobacteria* are, the more phycobilisomes they produce.

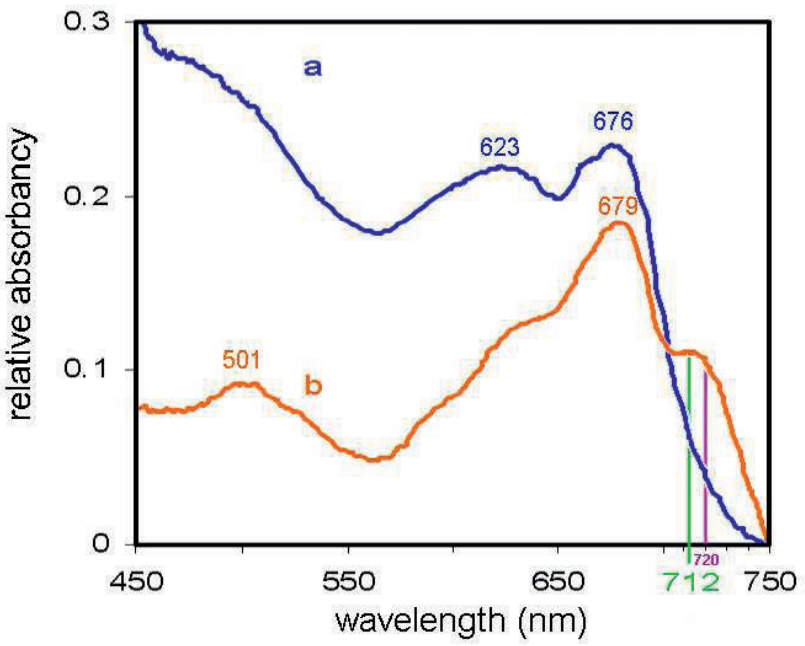

Fig. 15. Two *in vivo* reflection spectra of the middle of endolithic bands. (a) A site with high phycobilin content but no "bacteriochlorophyll-720". (b) A site with low phycobilin content but with "bacteriochlorophyll-720" (Horath et al., 2006).

Also more towards the interior of the rock, some spectra showed a shoulder between 712 nm and 720 nm. Decomposition into different Gaussian curves could give the real maximum absorption wavelength. An *in vivo* maximum absorption in this range is not common, but BChl *e* could fit with its literature value of λ_{max} between 715 and 726 nm (Gloe et al., 1975).

4.6. Structural studies

4.6.1. Scanning electron microscopy (SEM)

The preparation of samples for scanning electron microscopy is always confronted with the question of how much will the preparation alter the biological fabric with drying and surface coating. Samples from similar habitats like the ones of Dong et al. (2007) or of Friedmann (1974) show similar structures with ours. They also contain microbial structures such as globules, rods and filaments (Fig 16 and 17).

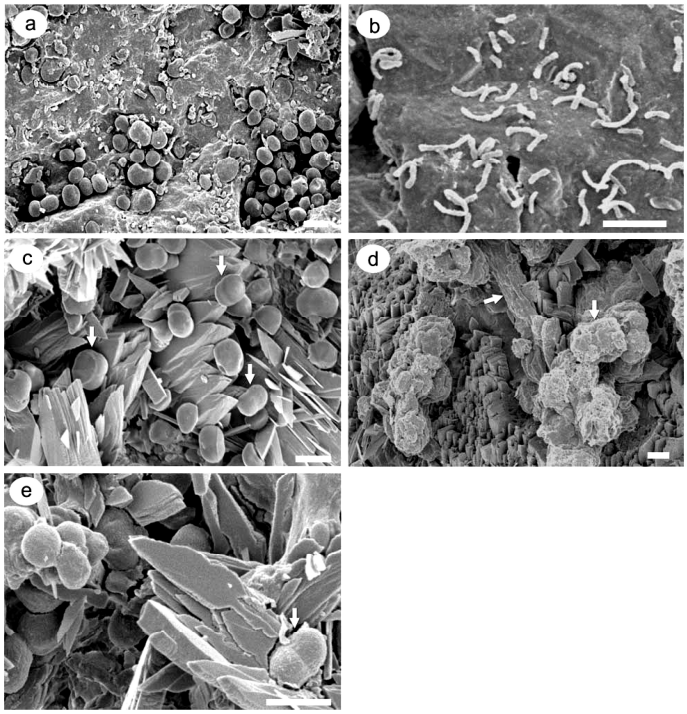

Fig. 16. SEM of fractured soil gypsum from (a) Al-Jafr (Jordan), with unicellular cyanobacteria and coccoid bacteria; (b) Al-Jafr (Jordan), with rod-shaped bacteria; (c) Atacama site (AT326b, Chile), with unicellular cyanobacteria and cell divisions (arrows); (d) Mojave (USA), with unicellular and filamentous cyanobacteria covered by exopolymer polysaccharide (arrows); (e) Al-Jafr (Jordan), with unicellular cyanobacteria with crosscutting relationship between the cyanobacteria displaying cell division (arrow, lower right) and a cluster of tabular pseudohexagonal gypsum crystals indicating dissolution of gypsum crystals during growth of the cyanobacteria. Scale bar is 5 μm (Dong et al., 2007).

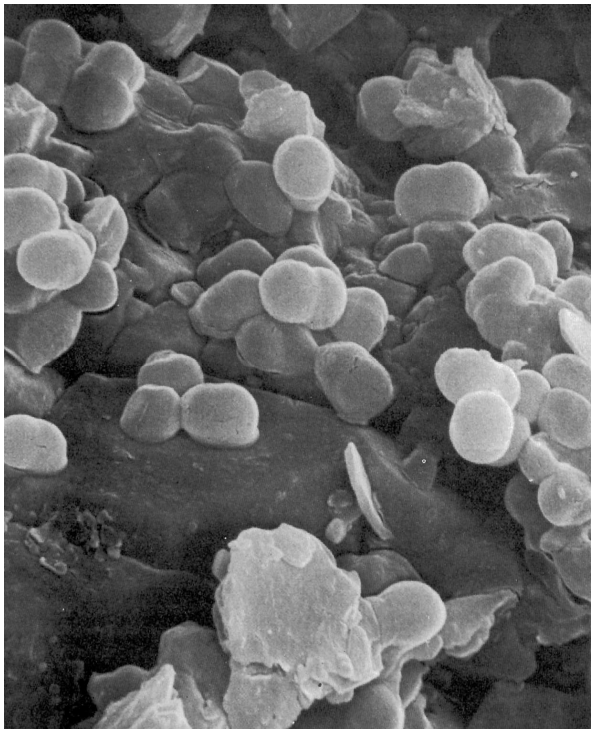

Fig. 17. SEM of endolithic algal growth in sandstone from the Negev Desert, near Nahal Timna. (Friedmann & Galun, 1974).

4.6.2. Confocal laser scanning microscopy (CLSM)

In most of the CLSM images three features are evident i) that the autofluorescent cells are clustered mostly as oval shaped colonies, ii) the colonies are inside a skin-like sheath

which is autofluorescent, iii) the cells produce a considerable amount of extracellular polymeric substances (EPS) around them. The glycoconjugates of these EPS were stained with the lectin of *Aleuria aurantia* labeled with Alexa-488 (fig. 9c in Horath et al. 2006). EPS not reacting with the lectin is not visible, but can be guessed around the autofluorescent cells. Different EPS matrices must be present, reacting differently with the specific *Aleuria* lectin. Figure 18 shows a group of chlorophyll-containing cells within a protecting envelope that emits a green autofluorescence after excitation with the two photon technique at 780 nm. The green color indicates fluorescence in the range of 400 to 552 nm. The image shows both, the autofluorescence of the sheath and DAPI staining of the cyanobacteria. The autofluorescence of the sheath was confirmed in unstained samples. The sheath may function as UV-shield and its fluorescence could be produced by the autofluorescence of Scytonemin. The depicted colony is indicative for *Gloeocapsa*.

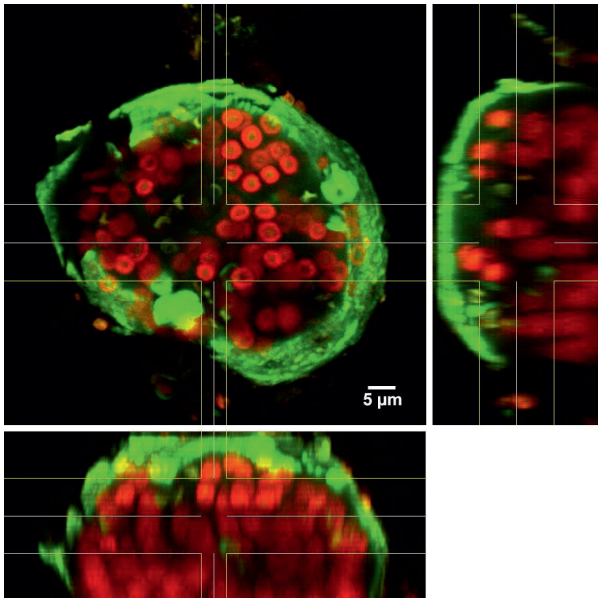

Fig. 18. CLSM image of a DAPI stained rock sample. Horizontal projection, and two cross sections. Autofluorescent cells (red) embedded in a large surrounding sheath that also shows autofluorescence (green). DAPI bound to DNA is visible in the centre of the cells. Data recorded with a 63x NA 0.9 water immersible objective lens. Detection windows are: 400–552 nm (DAPI and sheath) and 600–752 nm (chlorophyll). Excitation with IR (two photon technique) at 780 nm [rb129ii (25.6.2003)].

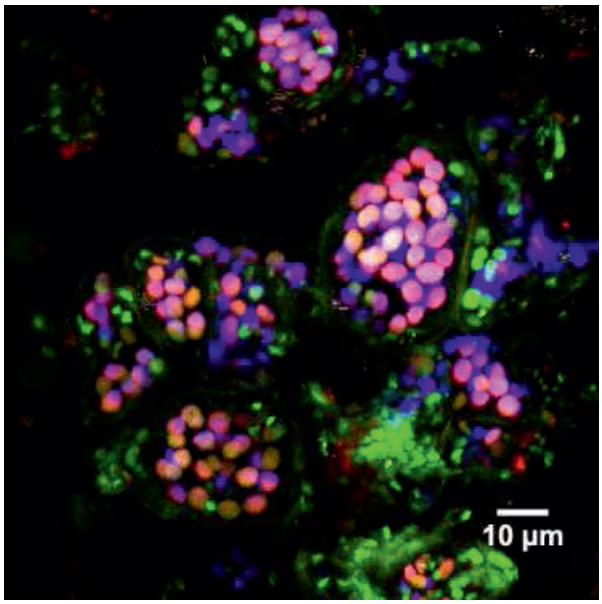

Fig. 19. Fixed rock sample showing autofluorescence in three emission windows. Image taken with a water immersion objective 63 x NA 1.2 and two photon excitation at 800 nm. Detection windows: 400–500 nm (green), 570–650 nm (red, phycocyanins), 670–800 nm (blue, chlorophyll) [rb025iiipr (12.12.2000)]

Figure 19 shows several colonies of cells that are fixed with paraformaldehyde. The detection windows are set to: a) 400–500 nm (green) representing an unknown autofluorescence; b) 570–650 nm (red) covers the autofluorescence of phycocyanins; c) 670–800 nm (blue) indicating the fluorescence of chlorophylls. Cyanobacteria appear usually in pink as a combination of the red (phycocyanin) and blue (Chl a) channel. Depending on light intensity and adaptation, the fluorescence of phycobilins is variable. Phycobilins show a high fluorescence when the energy transfer to the chlorophyll is overloaded because of high intensities of light. Some sheaths are visible in the green channel as well, indicating autofluorescence in the range of 400 to 500 nm (Fig.19).

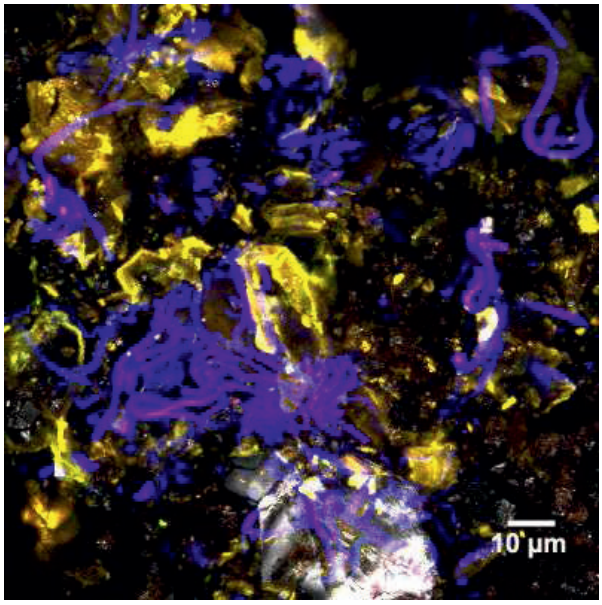

Fig. 20. Autofluorescence of filamentous phototrophic microorganisms (pink/blue). Data recorded with a water immersible objective: 63x NA 0.9. Excitation wave lengths = 488 nm, 568 nm, 633 nm. Detection windows: 480-495 nm, 500-550 nm, 575-625 nm, 650-800 nm [rb055fxyz (23.6.2003)]

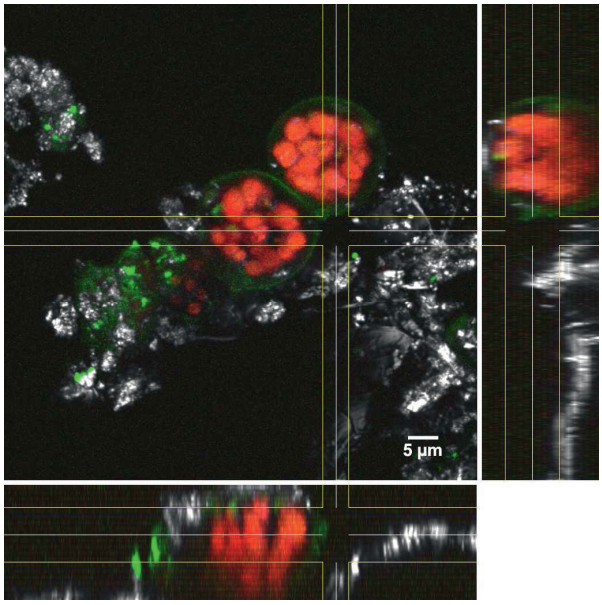

Fig. 21. CLSM image recorded sequentially: first with dual channel two photon technique, then with 1-photon reflection. The image shows a horizontal projection, and two cross sections. The two globular microcolonies show autofluorescence of phototrophic pigments (red) and a very thin sheath around them (faint green). Syto-40 cell staining (bright green) revealing also non-phototrophic organisms. Reflection indicates the mineral surface (white). Detection: 2-photon: 400-553 and 600-753, 1-photon: 480-500. Objective lens 63 x NA 0.9. [rb125iif, 25.6.2003]

Figure 20 shows an example with filamentous phototrophic microorganisms. The color allocations were as follows: white (reflection); pink/blue (autofluorescence), yellow

indicates a nonspecific overlay from two channels. The filamentous cells may be Chloroflexi as identified by molecular methods.

In figure 21 a rock sample stained with Syto-40 (excitation at 420 nm, fluorescence at 440 nm) is depicted. The dual channel 2-photon emission signal shows autofluorescence originating from phototrophic microorganisms in red (600–753 nm). The green 2-photon emission signal (400–553 nm) shows two features: in bright green the Syto-40 stained heterotrophic bacterial cells and in faint green the autofluorescence of the sheath surrounding the phototrophic microcolonies. The 1-photon reflection signal of the Piora dolomite is shown in white (480-500 nm).

5. Conclusions

The data presented in this thesis and in an increasing amount of publications show, that despite of extreme environment and conditions, Dolomite rock is a colonizable habitat which provides a niche for a broad and diverse spectrum of living microorganisms, for specialists at low light regimes for instance. It becomes clear that we still see only a small part of the natural system so far. New techniques like 454 sequencing will enlarge the picture and new organisms will be found. Recently the metagenome of ice from a glacier resulted in a large diversity not thought before (Simon et al., 2009). Pointing et al. (2009), using classic sequencing suggested in the abstract: "The findings show that biodiversity near the cold-arid limit for life is more complex than previously appreciated, but communities lack variability probably due to the high selective pressures of this extreme environment." Pasic et al. (2010) as well with classic sequencing mentioned "A total of eight bacterial phyla were detected. The application of various species richness estimators confirmed the diverse nature of the microbial community sample." The question of how many species are around on this world has been raised by Dykhuizen (1998) and Curtis et al. (2002), suggesting that "thirty grams of forest soil contains over half a million species" with the explanation: "I suppose that the explanation for such a large number of bacterial species is simply that speciation in bacteria is easy and extinction difficult." This will hold also for the endolithic environment. Though there are very frequent and dominant strains, the challenge will be to find the rare biome.

6. Outlook

In what direction will the research on endolithic organisms go? Is cultivation of endolithic organisms possible? Preliminary experiments with dolomite fragments in 10 to 1000 times diluted BG-11 or LB medium suggest a very limited growth induction by the addition of nutrients. No turbidity increase in LB was observed, however, the green of the endolithic band became more intense with time. Concerning cultivation, organisms in biofilms often behave like tissue cells of higher organisms. They may have adapted to their environment in a way that they become uncultivable under laboratory conditions or they may become metabolically inactive when their symbiotic associations are destroyed (Pohl et al., 1999). To produce pure cultures the mechanical separation of cells is a promising method. The isolation of pure cultures may be difficult but should be possible if the appropriate nutrients are supplied in the right amount and the drop-off products removed.

For the verification of the presence and for the spatial localization of Chloroflexi strains, various heterotrophs, and other microbes in the stone, *in situ* hybridization (FISH) could be a solution. However, the binding of the dye to Dolomite particles must be eliminated first or the stone material must be dissolved for example with EDTA before the hybridization. On the *in situ* physiological activity of the community, so far we have no data. No activity tests have been applied to quantify the metabolic state of the organisms embedded in the rock, as e.g. the photosynthetic activity of the cyanobacteria or the respiration of the heterotrophs. Such measurements could be used also to test the viability of endolithic samples after stress treatments like high irradiation or low gravity in simulations of extraterrestrial life in outer space.

7. References

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ENDOLITHIC MICROBIAL POPULATIONS IN DOLOMITE ROCK

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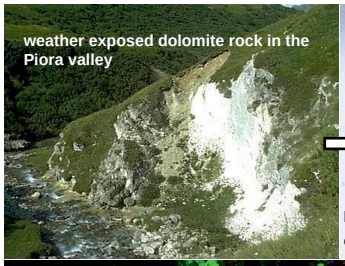
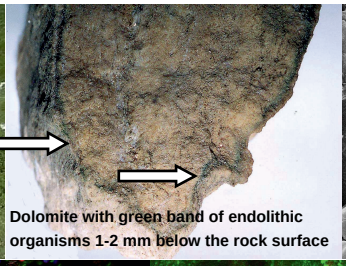
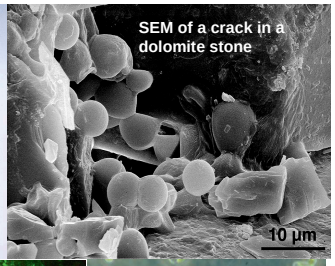
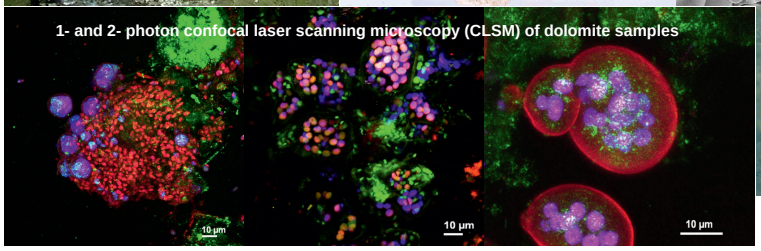
Endolithic microorganisms have been found in weather exposed rocks in dolomite formations in the southern part of the Alps (Piora, Ticino, Switzerland), visible as a green to brown band only a few mm below the rock surface. Cut and polished rock fragments were used for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Fine-grainy stone material has been used for DNA extraction and enrichment cultures.







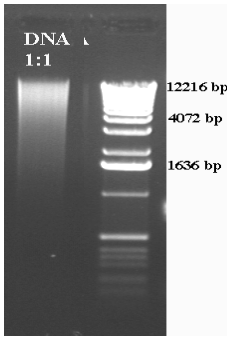


1-photon CLSM of EUB-CY3 labeled bacteria (red), autofluorescence from cyanobacteria (pink) and reflection signals (green)

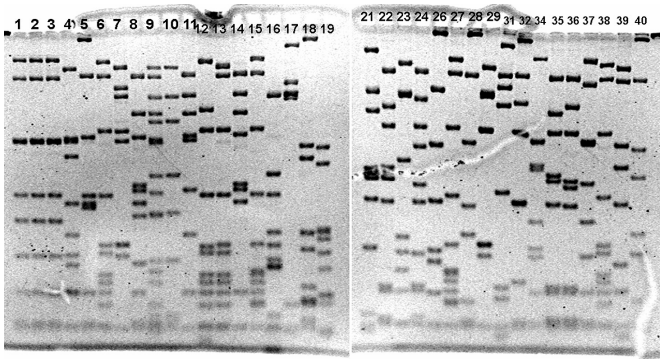
2-photon CLSM of microcolonies, autofluorescence: green=400-500nm (unknown), red=570-650nm (phycobilins), blue=670-800nm (chlorophyll)

1-photon CLSM of a cyanobacterial microcolony (pink) surrounded by an unknown layer (red) and reflection signals (green)

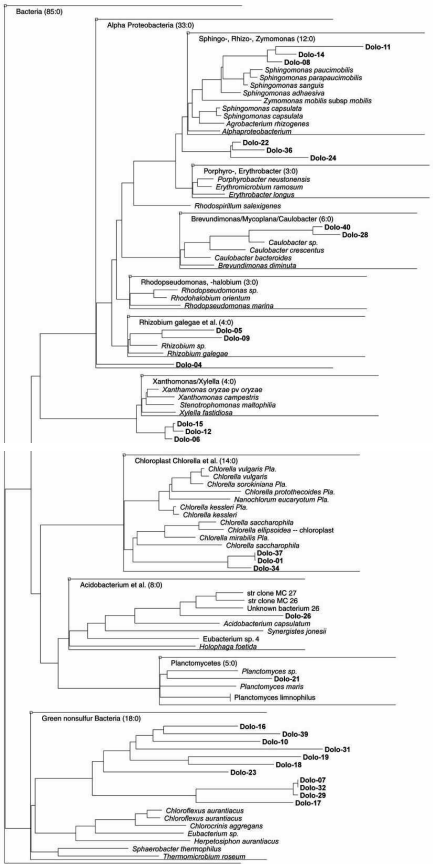
Using rock material as inocula into diluted BG-11 media, cyanobacteria like *Synechocystis* sp.and *Phormidium* sp., and the green alga *Stichococcus bacillaris* became enriched.



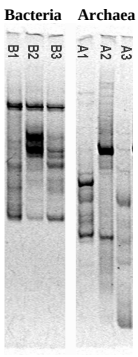
DNA was extracted from greenish-brown rock material using a bead beater and detergents (50 mM NaCl, 50mM EDTA, 50 mM Tris, 5% SDS, pH 8).

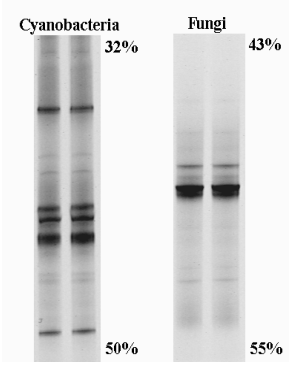


After DNA extraction, the 16S rDNA was amplified by using the bacterial primers S-D-Bact-0008-b-S-20 and S-K-Prok-1524-a-A-18 and cloned using the TOPO TA cloning kit. An RFLP with the enzymes *Hin*f I and *Hae* III was used to select the different inserts for sequencing. From 40 clones 4 did not contain any insert, 28 turned out to be different.



Phylogenetic tree of dolomite clones (Dolo-zz) generated with the ARB program (www.arb-home.de). There is a remarkable accumulation of sequenced 16 S rRNA genes in the phylum of Chloroflexi (group of Green Nonsulfur Bacteria).





For TTGE (54°to 70°C at 2°C/h and 6 V/cm, 6% (w/v) polyacrylamide, 7M urea, 1x TAE) extracted DNA was amplified with the primers S-D-Bact-0341-a-S-17 (**Bacteria**; B1, B2, B3) or S-D-Arch-0345-b-S-20 (**Archaea**; A1, A2, A3) and S-*-Univ-0907-GC-A-20+40. The numbers represent three different samples (1-3). For DGGE (**Cyanobacteria** and **Fungi,** denaturing gradient indicated, 100% denaturing solution = 40% (v/v) formamide and 7M urea) cyanobacterial or fungal primers have been used. Unique phylotypes were sequenced and indicated a variety of cyanobacterial species which confirmed the results of the PAGE-gels that show quite a distinct microbial diversity.

used primers:

S-D-Bact-0008-b-S-20	5'-AGA GTT TGA TCM TGG CTC AG-3'
S-K-Prok-1524-a-A-18	5'-AAG GAG GTG ATC CAR CCG-3'
S-D-Bact-0341-a-S-17	5'-CCT ACG GGA GGC AGC AG-3'
S-D-Arch-0345-b-S-20	5'-CGG GGY GCA SCA GGC GCG AA-3'
S-*-Univ-0907-GC-A-20+40	5'-(CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) CCG TCA ATT CMT TTR AGT TT-3'

Conclusions:

Combined microscopical, culturing and molecular techniques demonstrate a rich and specific endolithic microbial community composed of archaea, (cyano-) bacteria, and fungi in dolomite rock walls in the Piora valley in the Alps at 2000 m above sea level.

International Symposium on Subsurface Microbiology
Copenhagen, 8th - 13th of September 2002

ENDOLITHIC POPULATIONS IN DOLOMITE ROCK

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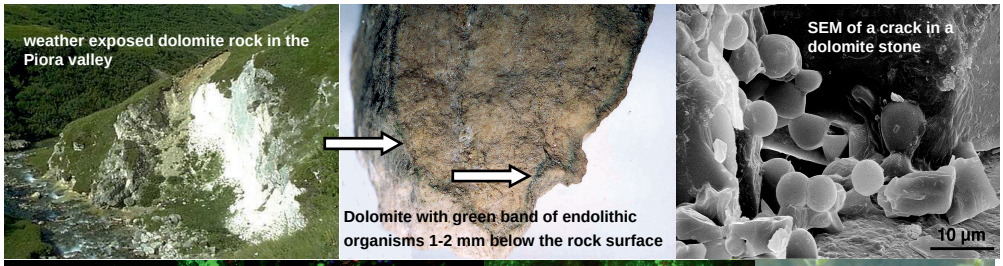
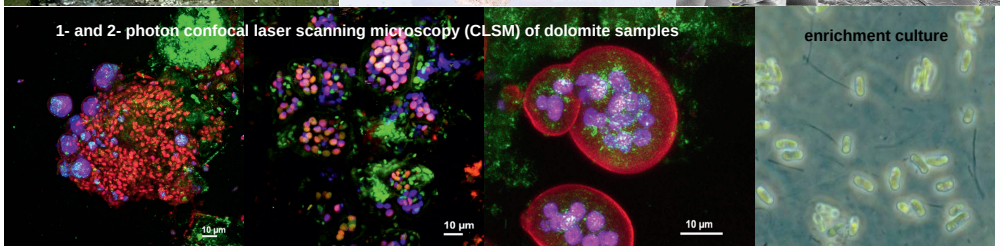
Endolithic microorganisms have been found in weather exposed rocks in dolomite formations in the southern part of the Alps (Piora, Ticino, Switzerland), visible as a green to brown band only a few mm below the rock surface. Cut and polished rock fragments were used for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Sugary-grained stone material has been used for DNA extraction and enrichment cultures.



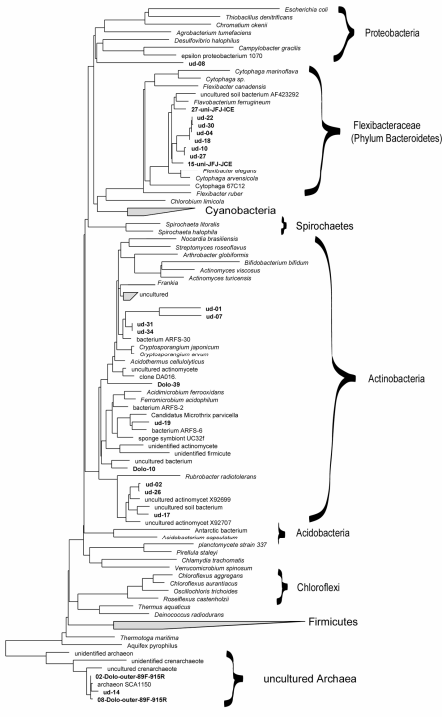


1-photon CLSM of EUB338-CY3 labeled bacteria (red), autofluorescence from cyanobacteria (pink) and reflection signals (green)

2-photon CLSM of microcolonies, autofluorescence: green=400-500nm (unknown), red=570-650nm (phycobilins), blue=670-800nm (chlorophyll)

1-photon CLSM of a cyanobacterial microcolony (pink) surrounded by an unknown layer (red) and reflection signals (green)

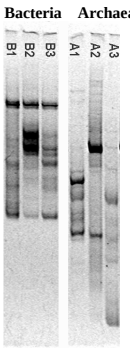
Using rock material as inocula into diluted BG-11 media, cyanobacteria like *Synechocystis* sp.and *Phormidium* sp., and the green alga *Stichococcus bacillaris* became enriched.

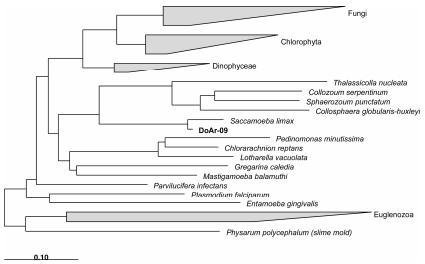


DNA was extracted from greenish-brown sugary-grained dolomite according to Sigler & Zeyer (2002) using a bead beater (Fast-prep, BIO 101), extraction buffer (50 mM NaCl, 50 mM EDTA, 50 mM Tris, 5% SDS, pH 8), and two sizes of glass beads (0.1 and 0.5 mm in diameter).

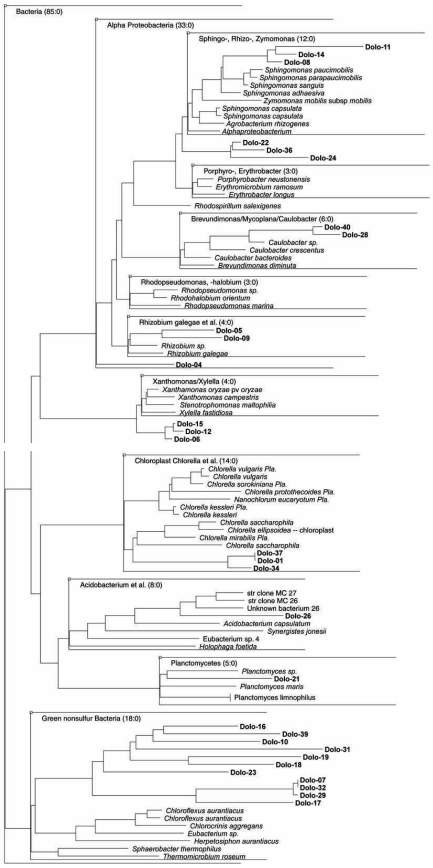
For TTGE (54° to 70°C at 2°C/h and 6 V/cm, 6% (w/v) polyacrylamide, 7M urea, 1x TAE) extracted DNA was amplified with the primers Bact-341s (**Bacteria**; B1, B2, B3) or Arch-345s (**Archaea**; A1, A2, A3) and Univ-907as-GC. The numbers represent three different samples (1-3).

For sequencing the 16S rDNA was amplified with different pairs of primers and cloned using the TOPO TA cloning kit. RFLP with the enzymes *Hinf* I and *Hae* III was used to select the different plasmid inserts.





Phylogenetic relations of plasmid inserts amplified from extracted DNA with universal primers (uni519s / uni1392as) named ud-##, specific archaeal primers (Arch-89F/Arch-915R), unspecific archaeal primers (Arch-0008s / Arch1517as, marked DoAr-##) or bacterial primers (bact-0008s/Prok-1524as; Dolo-##). After finding different bacteria (Dolo-##) we went searching for Archaea which also lead to the detection of new Eukarya (e.g. DoAr-09) because one of the used primer pair also fits to some of the eukaryotic small subunit ribosomal DNA. Most of the "ud"-clones file into the large phylum of Actinobacteria or class with the Flexibacteraceae from the Bacteroidetes phylum. Interestingly the cloned archaeal sequences neither group with the phylum Crenarchaeota nor with the phylum Euryarchaeota but with the slightly separated sequences of not yet cultivated Archaea.



Phylogenetic tree of dolomite clones (Dolo-##) fetched with the primers Bact-0008s and Prok-1524as. The cloned sequences line up with different groups such as the Alpha and Gamma (Xanthomonadaceae) Proteobacteria, chloroplasts of algae (*Chlorella saccharophila* chloroplast), Acidobacteria or Planctomycetes. A high accumulation of sequenced 16 S rRNA genes is found close to the phylum of Chloroflexi (group of Green Nonsulfur Bacteria).

Conclusion:

Microscopical, culturing and molecular techniques demonstrate a rich and specific endolithic microbial community composed of often so far unknown archaea, (cyano-) bacteria, fungi and other small eukaryotes like amoebae and mosses in dolomite rock walls in the Piora valley in the Alps at 2000 m above sea level.

Outlook:

To proove the presence of the detected organisms in situ with fluorescently labeled oligonucleotide probes is challenging since the DNA probes easyly get stuck to the dolomite stone instead of to the ribosomal RNA. Probably the whole stone needs to be removed from the organisms before applying FISH.

used primers:

S-D-Bact-0008-b-S-20	5'-AGA GTT TGA TCM TGG CTC AG-3'
S-D-Arch-0008-e-S-16	5'-TCY GGT TGA TCC TSC C-3'
S-K-Prok-1524-a-A-18	5'-AAG GAG GTG ATC CAR CCG-3'
S-D-Bact-0341-a-S-17	5'-CCT ACG GGA GGC AGC AG-3'
S-D-Arch-0345-b-S-20	5'-CCG GGY GCA SCA GCC GCG AA-3'
S-*-Univ-0907-GC-A-20+40	5'-(CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) CCG TCA ATT CMT TTR AGT TT-3'
S-D-Arch-1517-A-16	5'-ATC CAG CCG CAG RTT C-3'
S-Univ-1392-A	5'-ACG GGC GGT GTG TRC-3'
uni 519s	5'-CAG CMG CCG CGG TAA TWC-3'
Arch-89F	5'-ACG GCT CAG TAA CRC-3'
Arch-915R	5'-GTG CTC CCC CGC CAA TTC CT-3'

Reference
W. V. Sigler & J. Zeyer (2002) Microbial Diversity and Activity along the Forefields of Two Receding Glaciers. Microb. Ecol. 43: 397-407

Annual Assembly of Swiss Society for Microbiology
Lugano, 11th - 12th of March 2004

An endolithic habitat in Dolomite rock in the Alps

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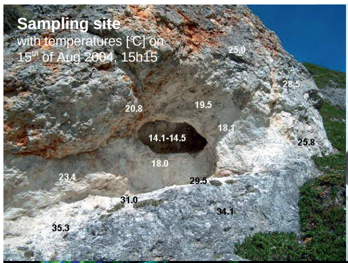
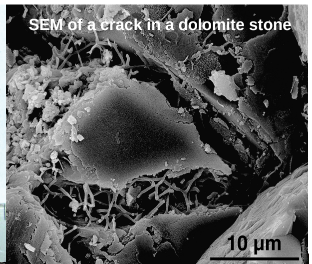
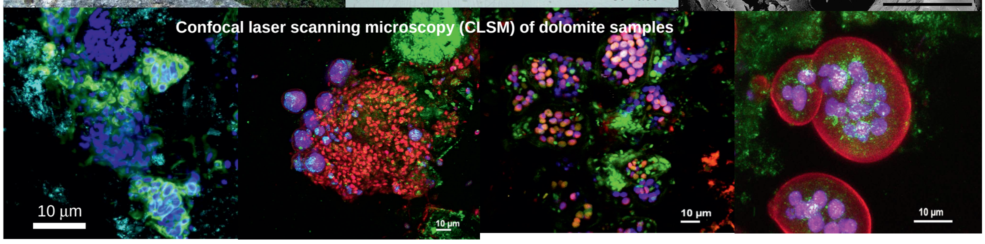
Endolithic microorganisms have been found in bare exposed rocks of dolomite in the southern part of the Alps (Piora Valley, Switzerland), visible as a green to brown band about 1-8 mm below the surface. Cut and polished rock fragments were used for reflectance spectroscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Material from the colored band was used for DNA extraction, 16S/18S rRNA gene cloning, and sequence analysis.





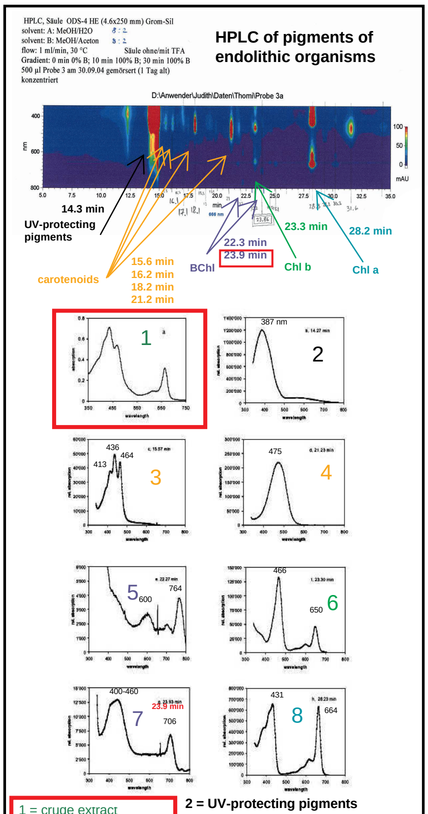


Autofluorescence from cyanobacteria (blue) and Alexa-488- *Aleuria aurantia* lectin fluorescently stained exopolymers (bright green)

EUB338-CY3 labeled bacteria (red), autofluorescence from cyanobacteria (pink) and reflection signals (green)

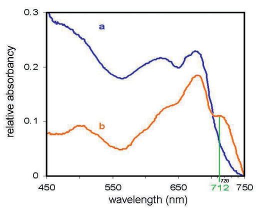
Microcolonies. Autofluorescence: green=400-500nm (unknown), red=570-650nm (phycobilins), blue=670-800nm (chlorophyll)

cyanobacterial microcolony (pink) surrounded by an unknown layer (red) and reflection signals (green)

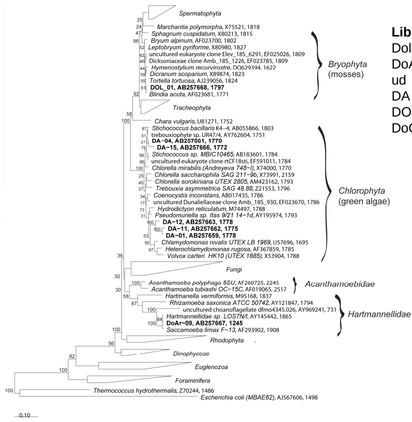


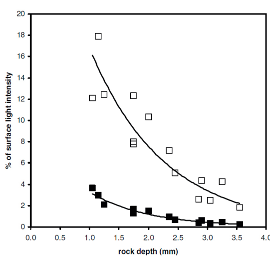
1 = crude extract (in Aceton : H_2O = 9 : 1)

2 = UV-protecting pigments
3 & 4 = carotenoids
5 & 7 = bacteriochlorophylls
6 = chlorophyll b
8 = chlorophyll a
(in Methanol : Aceton ≈ 8 : 2)



In situ reflection spectra from the center of endolithic bands. (a) Site with high phycobilin content, but no "bacteriochlorophyll 720". (b) Site with low phycobilin content, but with "bacteriochlorophyll 720". (phycoerythrin: 540-570nm, phycocyanin 580-620nm)





Light attenuation by Dolomite rock in % of surface light intensity. Spheres and squares: illumination with perpendicularly oriented bundled, and diffuse light, respectively.

Table 1. Selected biological and chemical parameters of the dolomite investigated in this study. Parameters were measured in bulk dolomite according to Fig. 1 unless otherwise noted.

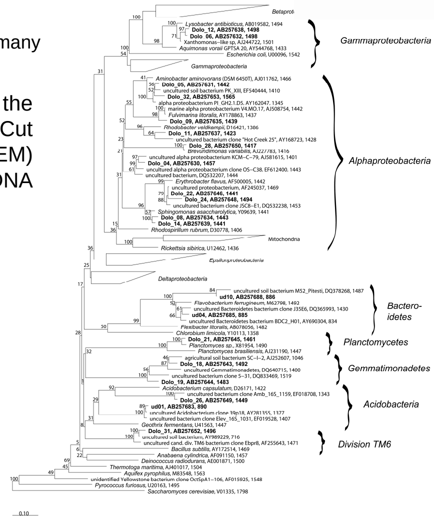
Parameter		Measured value
Total cell count:	red dolomite	4.0×10^9 (±1.1)[a]
	green dolomite	3.7×10^9 (±0.4)[a]
pH (in water)		9.5
pH (in $CaCl_2$)		8.5
C_{org}		1.90%
N_{tot}		0.02%
Ca		20.15%
Mg		11.67%
K		0.19%
Fe		0.19%
P		<0.002%
S		156 µg g^{-1}
Cl		95 µg g^{-1}

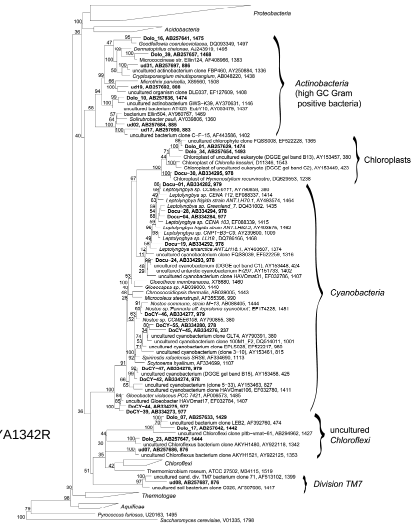
a. Calculated as the number of DAPI stained cells g^{-1} dry dolomite according to the method of Zarda *et al.* (1997).

Composition of Dolomite rock in the Piora Valley.

Sigler et al. (2003) *Environ Microbiol* **5**:618–627

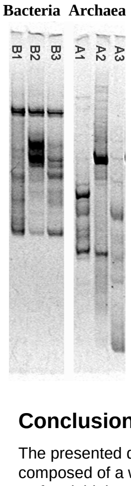
CAREX* Summer School ***June 28-July 3, 2010*** **in Pieve Tesino, Italy.**
(*coordination action for research activities on life in extreme environments)



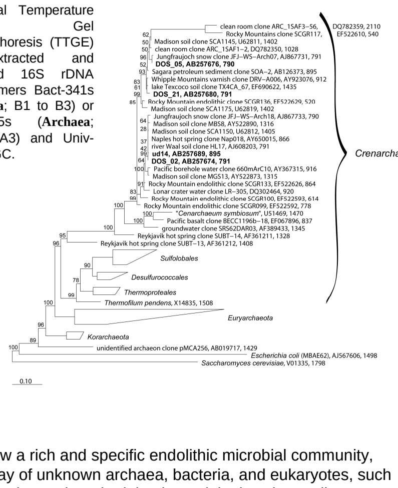


Library	Used primers
Dolo	27f - 1524r
DoAr	8aF - 1517r
ud	536f - 1392r
DA & DOL	8aF - 1512uR
DOS	89Fb - 915R
DoCY & Docu	CYA359F - CYA1342R

Phylogenetic trees of dolomite clones fetched with different primers (see list above). The two trees above are "bacterial". The cloned sequences line up with different groups such as the *Cyanobacteria*, *Actinobacteria* (high GC Gram +), *Alpha* and *Gamma Proteobacteria*, *Bacteroidetes*, *Acidobacteria* (Eventually some are also phototrophic: Bryant et al. 2007, *Science* **317**:523-526), chloroplasts of algae (*Chlorella saccharophila*), and *Planctomycetes*. Of the Green Nonsulfur Bacteria some strains of the uncultured *Chloroflexi* got detected.
More clones were obtained using "universal" primers (536f / 1392r), specific archaeal primers (89Fb, 915R), and unspecific archaeal primers (8aF, 1517r). The latter resulted in the detection of an organism close to the eukaryote *Saccamoeba limax (AF293902)* (tree at the left). The archaeal sequences found, group with the phylum "uncultured *Crenarchaeota*" where *Cenarchaeum symbiosum (U51469)* is the only cultivated one so far (tree below).



Temporal Temperature gradient Gel Electrophoresis (TTGE) of extracted and amplified 16S rDNA with primers Bact-341s (**Bacteria**; B1 to B3) or Arch-345s (**Archaea**; A1 to A3) and Univ-907as-GC.



Conclusion:

The presented data show a rich and specific endolithic microbial community, composed of a wide array of unknown archaea, bacteria, and eukaryotes, such as fungi, higher algae, and amoebae, in dolomite rock in the Piora valley at 2000 m above sea level. Recent literature showed that with different primers, for example shorter ones (Isenbarger et al. (2008) *Appl Environ Microbiol* **74**:840–849), also a broader or different diversity can be observed. We conclude that the detection of the microbial diversity in the endolithic habitat is still far from being exhausted.